Membrane sulphydryl groups in the control of water and ion balance in the red blood cell of the eel Anguilla anguilla L

El Missiry, M. A.

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MEMBRANE SULPHYDRYL GROUPS IN THE CONTROL OF WATER AND ION BALANCE IN THE RED BLOOD CELL OF THE EEL ANGUILLA ANGUILLA L.

Submitted by

M.A.EL MISSIRY

For

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M. A. EL MISSIRY

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

For my parents, my wife and

my sons, Khaled and Sherif.

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CONTENTS

ABSTRACT:

Sulphydryl groups are the main controlling sites for water and ion movement across cell membranes. There are two populations and these differ in both location and reactivity. This work shows that the organic mercurial compounds that specifically react with such groups, have differing effects on eel compared to human membranes. While there is an increase in the activation energy for osmotic water permeation in human on treatment with pHMB there is a decrease in that for eel. The rate of water diffusion, as measured by the water exchange time T_2, is decreased in human and increased in eel under pCMBS treatment. These effects on eel and human are explained in terms of a conformational change in the geometry of the aqueous channels leading to their virtual closure (in human) or opening (in eel) through specific reaction of pHMB or pCMBS with -SH groups in membrane proteins participating in channel structure.

20% of the sulphydryl population on the human erythrocyte membrane are responsible for controlling 80% of water transport are NEM-insensitive and are located close to the external surface. The rest (80%) are internally sited and are NEM-sensitive.

In eel cells the -SH groups are equally divided between the internal and external surface. Those internally embedded are NEM-insensitive, unlike human, and are responsible for 90% of water permeation control. The external -SH groups are NEM-sensitive.

pCMBS influences ion movements in a similar way in both eel and human red cells. It initiates K^+ loss and Na^+, Cl^- and water gain

eventually producing lysis. The effects are immediate in eel but delayed by 10 hrs in human. Analysis of cellular ion contents shows an increase in the osmotic elements in eel cells 1 hr post treatment continuing until haemolysis occurs after 2.5 hrs. Insignificant changes of cellular osmotic elements has occurred 6 hrs post pCMBS treatment of human cells. The addition of cysteine up to 2.5 hours post treatment in eel and human can reverse these ion changes. Later addition fails to produce recovery and eventual haemolysis occurs.

Ionizing radiation and organic mercurials appear to act on the same sulphydryl groups in both cell types. Exposure to doses up to 400 Gy oxidizes -SH groups in both eel and human red cell membranes. Human -SH groups are more radiosensitive, the D_{37} being 232 Gy while for eel it is 490 Gy. As a result of radiation exposure the osmotic water movement in both eel and human is immediately increased and the pattern of both -SH disappearance curves and increased water permeation provides support for the presence of two classes of membrane -SH groups in eel and human.

Radiation, like organic mercurials, produces similar effects on ion movement in eel and human cells and both have energy demanding mechanisms for recovery from the radiation effect with the eel systems being more efficient.

Units

All units in this work are quoted in Standard International (SI) units with the exception of osmolality. Traditionally, osmolality has been expressed as mosm Kg^{-1} and this tradition has been retained in the work although the Commission on Clinical Chemistry of the International Union of Pure and Applied Chemistry (IUPAC) and the International Federation of Clinical Chemistry (IFCC) have recommended that the unit of osmolality be m mol Kg^{-1}. It was felt that such an adoption at present would generate confusion. As an example, the concentration of a 1 Molal solution of NaCl (58.5gm NaCl Kg^{-1} (H_2O)) and written as a concentration of 1 mol Kg^{-1} (H_2O) will have an osmolality of 1.86 osm.Kg^{-1} (traditional units) or an osmotic concentration of 1.86 Molal (SI units). To differentiate between molalities in the new units, care must be exercised to stipulate that it is the actual osmotic concentration that is being discussed.

ABBREVIATIONS

Bromomercuric-2-hydroxypropane	BMHP
Chlormerodrin	CM
Dry cell solid	dcs
4, 4' diisothiocyano stilbene-2, 2'-disulfonate	DIDS
5, 5', Dithiobis-2-nitrobenzoate	DTNB
Ethylene diaminetraacetic acid	EDTA
Flourescein mercuric acetate	FMA
Glutathione (reduced)	GSH
Iodoacetamide	IAM
N-Ethylmaleimide	NEM
p-Aminophenyimercuric acetate	pAPMA
p-Chloromercuribenzoate Sulphonate	pCMBS
p-Hydroxymercuribenzoate	pHMB

CHAPTER 1

General Introduction

CHAPTER I

INTRODUCTION

A fundamental problem of animal and plant life is the regulation of the chemical activity of water inside the organism,since this limits the dissolved ions which in turn are critical for the maintenance of cell biochemistry.

Euryhaline fishes like trout and eel that migrate between salt and fresh water on a diurnal or seasonal time scale are subjected to osmotic stresses that have required the elaboration of osmotic and ionic regulatory mechanisms at both the whole organism and cellular levels. When fresh-water adapted rainbow trout are transferred directly to 32% sea-water, one of the earliest responses is an increase in the animal's drinking rate by fifty fold (Bath and Eddy, 1979). This is accompanied by a diffusion of ions down their concentration gradients into the blood via the gills with a subsequent increase in plasma and body salt content. Similar drinking responses have also been observed in the eel (Kirsch and Mayer-Gostan, 1973). Salmon parr, however, when placed in 34% sea water, show an immediate increase in plasma osmotic pressure and subsequent death because of a poorly developed regulatory mechanism (Parry, 1960).

At the cellular level, the major functions of the plasma membrane are to control the entry of cell nutrients and oxygen, the exit of wastes and the regulation of the ionic composition of the

intracellular fluid. Because this fluid contains solutes at concentrations that are usually quite different from their corresponding levels in the bathing medium, the steady state existence of the cell is critically dependent on the ability of its membrane to discriminate between various solutes (Sha'afi, 1981). The movement of water and solutes across the membrane is a dynamic process and the cell is only in equilibrium with its environment in terms of its contents when it is dead (Sha'afi, 1977).

With about 55 to 60% of an animal's body weight attributable to water and approximately two-thirds of this within cells, the constancy of body weight will be dependent on the precision with which cells can regulate both their water and ion contents (MacKnight and Leaf, 1978).

Because cell membranes are permeable to both water and ions, the net movement of water between cells and their extracellular fluids are as a consequence of the osmotic gradients across these compartments. If the osmolality of the extracellular fluids decreases, water moves into cells, thereby increasing their volume, whereas an increase in interstitial fluid osmolality would have the opposite effect, decreasing cell volume.

Of relevance to this work, Shawkat (1984) observed that the sudden immersion of eel erythrocytes into a hypotonic NaCl solution of 200 mosm Kg^{-1} resulted in a rapid influx of water (within seconds) and a subsequent volume control brought about by a furosemide inhibited K^+/Cl^- co-transport. The intracellular Na^+ ions appeared to have little or no function in such a mechanism.

Studies on the permeability of cell membranes have been of considerable importance in helping to define the functional and structural properties of the plasma membrane and the factors that determine the movement of different substances across the various tissues of the body. From such work it appears that the water flow rates across the cell membranes are extremely variable between animal species. Brahm and Wieth (1977) and Brahm (1982) found high water permeabilities in erythrocytes taken from human, dog, duck and pigeon and low values in those from chicken, giant salamander and foetal human. This is in spite of the similarities in structure of the integral membrane proteins of both chicken and human cells. Relatively high water permeabilities have also been found in certain fish species such as mackerel, bonito and hickory shad (Brahm and Wieth, 1977). Blum and Forster (1970) similarly showed that the water permeability of chicken and eel erythrocytes is considerably lower than that for human cells. At present it is not easy to suggest the adaptational significance of such variabilities.

Benesch and Benesch(1954) found that teleost fish erythrocytes are more susceptible to haemolysis by organic mercurials than mammalian cells (sheep) and first suggested the importance and a role for the membrane sulphydryl groups in the maintenance of functional and structural integrity of cell membranes. Their observations were followed by a report describing the close relationship between membrane sulphydryl groups and water and ion movement (Sutherland et al (1967), Macey and Farmer (1978), Conlon and Outhred (1978) and Brahm (1982)). All of these latter investigations focussed attention on mammalian cells especially human erythrocytes and ignored those of lower vertebrates.

Although much work has been done, the mechanisms controlling water and ion movement across the biological membrane is still far from clear and it is the purpose of this present study to provide further experimental evidence for the role of membrane sulphydryl groups in the mechanisms for water movement control across the surface of, in particular, lower vertebrate erythrocytes. A comparative study on these membranes with those of the much researched human cells was considered to be a valuable approach to the problem.

Work on red blood cells offers several advantages for such permeability studies. Not only does it permit a comparison of strictly homologous cells from many species, but they can be obtained, generally, in large quantities and with minimum treatment and can be kept in good condition outside the body for several days. A further advantage is that mature erythrocytes are completely separated anatomically from other cells and from each other and they may therefore be washed free from all extraneous materials that may influence their physiological state.

With human erythrocytes having been extensively investigated, and a need to examine the characteristics of other animal species, it was decided to study an animal that naturally undertakes both anadromous and catadromous migration in the hope that their cells, being more naturally placed under osmotic stress than mammalian ones, would have a more acutely developed mechanism for ion and water control.

Basic plan of the work:

The nature and the state of membrane-water interaction can be initially investigated by studying the energetics of water

permeation across erythrocyte membranes. In chapter 2 and extended in chapter 3 the temperature dependence of rates of osmotic haemolysis due to bulk water flow across both eel and human erythrocyte membranes was performed under normal conditions and under the influence of the specific sulphydryl reagents pHMB and its sulphonate derivative pCMBS, known water channel blockers. To compliment this, the effects of pCMBS on diffusional water flow was investigated using nuclear magnetic resonance to measure the water relaxation time (T_2). In this way the mechanisms and pathways controlling osmotic and diffusional flow could be compared.

^{203}Hg labelled pCMBS binding studies are described in chapter 4 to investigate the geographic location of functionally important sulphydryl groups on the eel erythrocyte membrane compared to those on human cells.

The changes in intracellular electrolyte and water content of cells under the influence of treatment with the organic mercurial compound pCMBS are presented in chapter 5 and in chapter 6, ionizing radiation was used as an investigational tool in an attempt to further characterize the nature of sulphydryl groups and their repair or replacement after damage.

Chapter 2

The temperature dependence of water flow across red blood cell membranes

2.1 INTRODUCTION

The functional state of water in biological membranes can, in part, be understood by investigating the energetics of water permeation across them and several articles have been published dealing with the measurement of activation energies for temperature dependent water fluxes (Hays and Leaf (1962a,b), Farmer and Macey (1970), Vieira et al, 1970). Although activation energies are useful for purposes of comparison between different membrane systems, caution is necessary in interpreting them in terms of molecular events. If two processes have the same mechanism of action, then their activation energies will be the same. The converse may obviously not necessarily be true. However in the case of movement of water across different membranes Colon and Outhred (1978) put forward evidence to suggest that similar values obtained for activation energies increase the likelihood that the mechanisms involved will be the same.

2.1.1 Theoretical Concepts

The self-diffusion of water molecules through water can be described by the diffusion coefficient, D_w. It is temperature dependent and obeys the classical Arrhenius equation:

$$D_w = D_o e^{-E_a/RT} \qquad (1)$$

where D_o is a constant termed the frequency factor, T is

the absolute temperature, R, the gas content and E_a the activation energy. To convert to a linear relationship equation 1 is logged:

$$\log_e D_w = \log_e D_o - E_a/RT \qquad (2)$$

so that a graph of $\log_e D_w$ v 1/T will give a negative slope of value E_a/R from which E_a may be calculated.

Another way of describing the temperature dependence of D_w, as well as the viscosity, η_w, is through the concept of the transition state theory of rate processes in which a water molecule moves by going through a transition state during the molecular jumping process of diffusion. The formation of this transition state is associated with both the activation energy and the entropy of activation. The temperature dependence of both self-diffusion and viscosity is governed by a relationship similar to equation 2, and it is usual for physiologists to consider only the activation energy E_a of the system as the critical parameter.

2.1.2 Diffusional permeability

Amphibian epithelial tissues have been used extensively for water permeation studies and the interpretation of activation energy values for them can help in interpreting blood cell data.

Hays and Leaf (1962b) calculated the apparent activation energy for the diffusion of tritiated water (THO) across

the unmodified membrane of isolated toad bladder to be 75 KJ mol^{-1} ; this value being much larger than the value 19.3 KJ mol^{-1} obtained by Wang et al (1953) for THO diffusion in bulk water and suggesting a restricted water pathway across the tissue.

Grigera and Cereijido (1971) studied the rate of water movement across the outer barrier of isolated frog skin and found that only above 25°C is the activation energy for the diffusion of tritiated water similar to that observed in free water. They further found that at a temperature of 15°C, the energy of activation is 35.58 KJ mol^{-1} and at a temperature near 0°C, at which the frog lives for part of the year, the energy of activation is 69.9 KJ mol^{-1}. They suggested that this low permeability of the outward facing membrane may be due to the fact that, at room temperature, it contains water in a non liquid state. An apparent activation energy closely related to that of THO diffusion in free solution, is generally taken as an indication that the water flux proceeds through a diffusional pathway containing water in free solution. Conversely an E_a larger than this value is assumed to indicate that the state of water in the membrane is more structured than in free solution and that energy has to be expended to create vacant lattice points where the migrating molecules could jump.

Vieira et al (1970) using a stopped flow technique studied water diffusion in human and dog red cell membranes and

found that the apparent activation energy for water diffusion permeability was 20.5 KJ mol^{-1} in dog and 25 KJ mol^{-1} in human over the temperature range 7°C and 37°C. In the case of dog red cells, the apparent activation energy for diffusion is similar to that for self-diffusion of water indicating a diffusional process equivalent to that in free solution. They also showed that the slightly, but significantly, higher activation energy for water diffusion in human red cell is consistent with a water/membrane interaction in the narrower equivalent pores characteristic of these cells (0.43 nm for human (Solomon, 1968) compared to 0.6 nm for dog red cells).

The temperature dependence of the water exchange time as measured by NMR through human erythrocyte membranes is characterised by values of apparent activation energies ranging between 25 and 33 KJ mol^{-1} (Morariu and Benga, 1977). These values are similar to the activation energies of those for either the molecular rotation or the diffusion of water in mono-or submonolayers of adsorbed water.

The temperature dependence of diffusional water exchange by NMR technique has also been used to study isolated human red cells (Morariu et al , 1981) and at pH 7.4 a definite break was found in the Arrhenius plot at around 26°C. The apparent activation energy at temperatures higher than the break was 23.8 KJ mol^{-1}, while at lower temperatures, the value was 5.8 KJ mol^{-1}. Recently Morariu et al (1985)

calculated an overall activation energy of 12.1 KJ mol^{-1} for water diffusion through unwashed blood containing a 5 mM concentration of Manganese (Mn^{2+}), while a value of 24.3 KJ mol^{-1} was obtained for whole blood with Mn^{2+} concentrations of 30 mM. In both cases no breaks or phase transitions were observed. In contrast, washed blood at 5 mM Mn^{2+} showed an activation energy of 21 KJ mol^{-1} without a phase transition while at 30 mM Mn^{2+} a phase transition was obtained at 28°C with E_a of 16.8 and 37.5 KJ mol^{-1} at the low and high temperature range respectively. Furthermore, their investigation showed that washed red cells, collected from infants less than 2 days old behave quite differently with respect to water diffusion when compared to adult erythrocytes. In such infant cells they showed Arrhenius plots consisting of two distinct lines with slopes of opposite signs and E_as of -2.1 ± 0.6 and $+ 3.9 \pm 0.4$ KJ mol^{-1} and concluded that neither these low positive or negative values of E_a could have any meaning for water diffusion exchange. It is unfortunate that these authors did not consider the implications of the problem further because the sophistication of the NMR technique has now raised questions on what may be a very important physiological mechanism in human cells.

Brahm (1982) calculated an apparent activation energy of 21 KJ mol^{-1} for water diffusion across intact human erythrocytes and 30 KJ mol^{-1} for red cell ghosts. He did not comment on the physiological significance of the difference for these values.

2.1.3 Osmotic Permeability

Several workers have studied the temperature dependence of osmotic water movement across different cell membranes in order to gain some insight into the nature of the membrane and in particular the state of water in that membrane. Table (I) shows some values obtained for the activation energies (E_a) for osmotic water flow in animal cells.

Table (I): A literature survey of activation energies due to osmotic permeability of water across different animal cells and tissues

Cell or Tissue	E_a(KJ mol^{-1})	Reference
Human Erythrocytes	13.8	Vieira et al (1970)
Dog Erythrocytes	15.5	Vieira et al (1970)
Cow Erythrocytes	16.7	Farmer & Macey (1970)
Chicken Erythrocytes	47.7	Farmer & Macey (1970)
Sheep Foetal Erythrocyte	31.8	Widdase (1951)
Sea Urchin egg	54-71	McCutcheon & Lucke (1932)
Barnacle muscle	31.4	Bunch & Edwards (1969)

As expected the larger activation energies are found in the relatively impermeable cells. Danielli and Davson (1935)

have envisaged the activation energy as a barrier to molecular motion and to overcome this barrier, each molecule had to acquire an energy greater than the activation energy. Accordingly, the lower the value for water permeability then the higher the corresponding value for the activation energy will be.

If water permeation across cell membranes proceeds through narrow pores containing highly ordered water, then the diameter of the pores may have a crucial effect on the value of the activation energy (House, 1974). Vieira, et al (1970) have examined that possibility by measuring E_a for both osmotic and diffusional permeability in human and dog erythrocytes. The diffusional permeability data has been reviewed previously. For osmotic water movement they calculated E_a for human to be 13.8 KJ mol^{-1} and 15.5KJ mol^{-1} for dog. These cells were chosen deliberately because the equivalent pore radius for dog erythrocytes is about 0.6 nm and that for human erythrocytes 0.44 nm. The E_a values for osmotic flow reflect this difference in pore size and in both cells are similar to the values reported for those related to the viscosity of water, ie 20 KJ mol^{-1} (Wang et al ,1953, Wang, 1965 and Mills, 1973).

As Poiseuille's law describes laminar flow through an equivalent pore, the osmotic permeability (P_{os}) will be equal to:

$$P_{os} = N \pi r^4 / 8 \eta_w \Delta X \qquad (3)$$

where N is the number, r the radius, ΔX the length of each of these pathways and η_w is the water viscosity within the pathways. This latter parameter should reflect the degree of water membrane interaction and in physical terms is the only temperature sensitive variable in the expression. House (1974) explains that if the quantity Lp η_w for a given membrane is invariant over a certain temperature range then one could conclude that osmotic flow is achieved by bulk flow through pores rather than diffusion. Although Vieira et al (1970) observed that for dog and human erythrocytes, Lp η_w was not independent of temperature, for them it provided strong support for the view that changes in temperature over the range 5 to 39°C does not affect the geometry of the equivalent pore as the Poiseuille coefficient ($r^4/\Delta X$) will also be constant. They concluded also that osmotic flow through the different equivalent pores of these two cells is indistinguishable from viscous flow in bulk.

A major conceptual problem is that the average activation energy for water diffusion across the human red cell (25 KJ mol^{-1}) is higher than that for both self diffusion and osmotic flow. Vieira et al (1970) have ascribed this disparity to a possible interaction between water molecules and the membrane. However it is hard to understand why such an interaction would not also influence E_a for osmotic water flow across the same membrane. Naccache and Sha'afi (1974), using the haemolysis technique, have also reported a low E_a for osmotic water movement through human

erythrocyte membrane (13.3 KJ mol^{-1}) and recently, Whittembury et al (1984) calculated the apparent activation energy for osmotic permeability of the basolateral plasma membrane of isolated proximal kidney tubules from rabbit to be 13.4 KJ mol^{-1}. This value, lower than that for bulk water viscosity, may indicate a continuum for water movement existing across the cell membrane and thus explain a low degree of interaction between water molecules moving across the membrane under the osmotic force and the membrane. They also suggested the possibility that a low E_a would be compatible with some form of molecular slip at the water-membrane interface in the water pore. A slippage at this interface was previously suggested by Vieira et al (1970) to explain the low activation energy of osmotic water movement across human erythrocyte membrane. Naccache and Sha'afi (1974), who obtained similar activation energies for osmotic water flow ignored the problem but Brahm (1982) while accepting that slippage could explain the low activation energy of water transport, indicated that, the slippage itself must be almost insensitive to temperature.

Interestingly, most of the estimates for the activation energy for osmotic water flow in other animal cells (Table I) exceeds the corresponding activation energies for viscous flow and self diffusion of water. For example Hempling (1973) produced values of 54-75 KJ mol^{-1} for leucocytes from a range of mammalian species and explained these high values as being due to water being intimately

associated with the outer boundary of the cell and quite sensitive to changes in membrane structure occurring in response to change in temperature, whether the mechanism is one of altered membrane solubility, chemical composition or change in the size of effective pores is yet to be decided.

2.1.4 Action of Mercurial compounds on the Activation Energy

Previous determinations for the temperature dependence of diffusional water permeability in human erythrocytes by means of tracer (Vieira et al (1970) and NMR techniques (Shporer and Civan (1975), Morariu and Benga (1977), Conlon and Outhred (1978) and Brahm (1982)) gave E_a values between 22 and 36 KJ mol^{-1}, whereas, the activation energy for osmotic water transfer through human erythrocyte (Vieira et al (1970) and Naccache and Sha'afi (1974)) and across basolateral plasma membranes of proximal tubules (Whittembury et al (1984)) gave values of 13-14 KJ mol^{-1}. Both of these values are changed by inhibition of water movement with pCMBS. The diffusion study by Conlon and Outhred (1978) showed an increase in activation energy from 22 KJ mol^{-1} to 40 KJ mol^{-1} and in Brahm's study from 30 to 60 KJ mol^{-1} after pCMBS treatment. This increase in E_a for water movement with pCMBS treatment has been seen in all tissues and cells studied (Naccache and Sha'afi (1974), Brahm (1982) and Whittembury et al (1984)). In contrast to the low activation energies of water transfer through human erythrocyte membranes, the E_a for water permeability through lecithin/cholestrol bilayers is high with values of

from 50 to 58 KJ mol^{-1} (Haydon (1969) and House (1974)). When mercurial reagents (like pCMBS) are applied to red cells, their water transport properties resemble the artificial lipid bilayer, ie water permeability decreases by a factor of ten (Macey and Farmer 1970) and the activation energy increases to 46 to 50 KJ mol^{-1} (Brahm, 1982). Relevant data are collected in table (II) for comparison between various activation energies under normal and pCMBS treated cells and lipid membranes.

Macey et al (1972) and Naccache and Sha'afi (1974) proposed that pCMBS blocks the protein pathway for water flow, leaving a lipid pathway only. This proposal was offered to explain the high activation energies obtained under pCMBS treatment which resemble those for artificial lipid membranes. Conlon and Outhred (1978) examined the activation energy of the putative protein pathway, by subtracting measurements for pCMBS-treated cells from those for untreated cells. They found an activation energy of 16.9 KJ mol^{-1} comparable with that for water self diffusion. Recently, a study of the thermodynamics of water channel closure in red cells was made assuming that, following maximal inhibtion by pCMBS, water permeation takes place exclusively through the lipid bilayer portions of the membrane. Based on this work Moura et al (1984) suggested that a more precise designation for the lipid pathway would be the pCMBS-insensitive and pCMBS-sensitive pathways. pCMBS-sensitive paths have characteristics commonly attributed to channels whereas pCMBS-insensitive paths do not.

Table II Water Transport Activation Energies of normal, pCMBS treated cells and Lipid membranes

Sample	E_a KJ mol^{-1}	Method	Reference
Human Erythrocytes	28.5	NMR	Conlon & Outhred (1978)
Human Erythrocyte + pCMBS	40.5	NMR	"
Human red cell ghosts	22	Tracer	Brahm 1982
Human red cell ghosts + pCMBS	60	"	"
Basolateral membrane of proximal tubule of kidney of rabbit	13	Osmotic	Whittembury (1984)
" + pCMBS	38.5	"	"
Human Erythrocyte	20	Osmotic	Macey et al (1972)
" + pCMBS	48	"	"
Human Erythrocyte	13	"	Naccache and Sha'afi (1974)
" + pCMBS	31.06	"	"
Red cell lipid	40	NMR	Conlon & Outhred (1978)
Red cell protein	16.9	NMR	"
Vesicle of Lecithin,	50	NMR	Quoted from Conlon & Outhred (1978)
Vesicle of dipalmitoyl Lecithin	63	NMR	"

2.2 MATERIALS AND METHODS

2.2.1 Materials

2.2.1.1 Erythrocytes

Erythrocytes from eel and human were used in this investigation.

Human blood was drawn from adult donors by venipuncture just prior to the experiment.

The Common Western eel, Anguilla anguilla, used for blood sampling, were caught in eel traps in the river Severn at Tewkesbury during 1983-86. The weight range was 200-700 gm and they were between 50-95cm long. They were maintained in large fresh water tanks at a temperature 11 $\pm$ 1°C in 12:12 light:dark regime. Most animals were 3-5 years old and hence were silver eels.

Eels were first stunned, cleaned of mucous then decapitated just behind the pectoral fins. Blood was collected directly from the heart into lithium heparinized tubes (15 u/ml). Oxalate was avoided because of its tendency to cause osmotic abnormalities of erythrocytes (Pounder (1944) and Jacobs et al (1950)). Neither clotting nor haemolysis was observed during the experiments.

2.2.1.2 Chemicals

Analytical reagent grade chemicals were used throughout.

Physiological Saline Solutions

A stock solution of concentrated buffered sodium chloride was made as follows: Sodium Chloride 90 gm; disodium hydrogen phosphate (Na_2 H PO_4) 13.65 gm, (if Na_2 H PO_4 - 2 H_2O, 17.15 gm) and sodium dihydrogen phosphate (Na H_2 PO_4 - 2 H_2O), 2.43gm were dissolved in distilled water and the final volume adjusted to one litre.

A physiological saline solution was freshly prepared using a 1:10 dilution of the buffered NaCl stock. KCl and glucose were added as necessary. The standard physiological saline used in the experiments consisted of 163 mM Na^+; 151 mM Cl^-, 11 mM HPO_4^{2-}, 6 mM K^+ and 5 mM glucose and was osmotically equivalent to 300 mosm Kg^{-1} and had a pH of 7.4

The organic mercurial para-Hydroxymercuribenzoate (pHMB) and parachloromercuriphenyl sulphonic acid (pCMBS) monosodium salt were used as membrane water flow inhibitors and obtained from Sigma Chemical Company USA. p-Hydroxymercuribenzoate is often called p-chloromercuri-benzoate because the chloro converts to hydroxy when the sodium salt is prepared. The inhibitors were freshly prepared for each experiment in phosphate buffered saline

solution before the addition to the buffered cell suspension. It was found that their effects on water transport across cell membrane increased with concentration up to about 1 mM and thereafter become concentration independent (Macey et al, 1972 and Brahm et al, 1976). One and two mM concentrations were used in temperature dependence experiments with eel and human erythrocytes respectively. In all cases, cells were pre-exposed to the mercurial compounds for at least 30 min before assay.

Cell Washing:

In experiments requiring washed erythrocytes, the blood was first subjected to a preliminary centrifugation at 450 x g for five minutes with subsequent removal of the plasma and buffy coat by aspiration. The cell fraction was washed three times with isotonic buffered saline solution, each involving centrifugation at 2000 xg for 5 minutes. The washed cells were finally suspended in the same isotonic saline solution at the desired haematocrit, generally 10%.

2.2.2 Analytical procedures

The rate of water entry into the cells was measured both under an osmotic gradient using the haemolysis technique and under steady state using the NMR - Relaxation time (T_2) technique.

2.2.2.1 Haemolysis Method (osmotic flow)

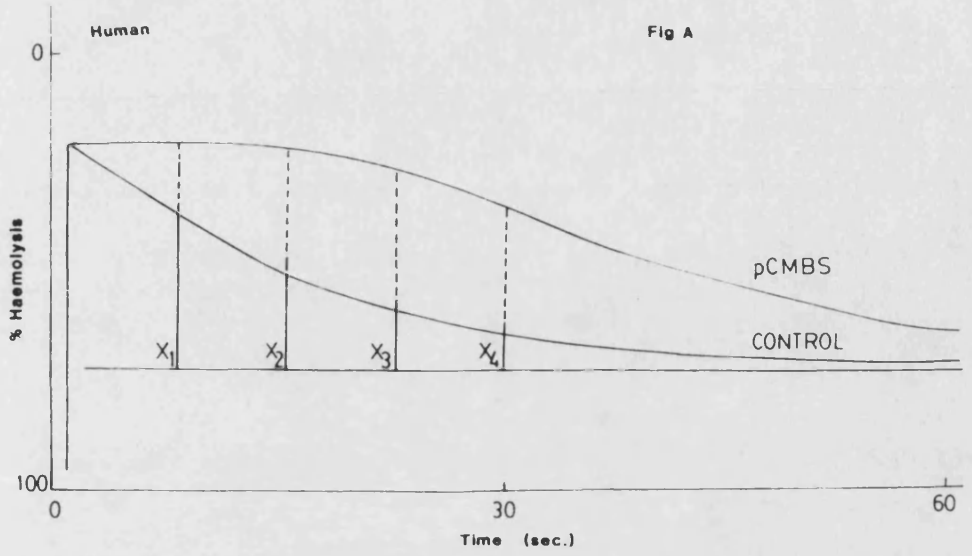
In this method the red cell suspension is subjected to a sudden decrease in osmotic pressure to produce 75% haemolysis. This end point is achieved in one minute and produced highly reproducible and consistent results (Jacobs et al, 1950 and Naccache and Sha'afi,1974).

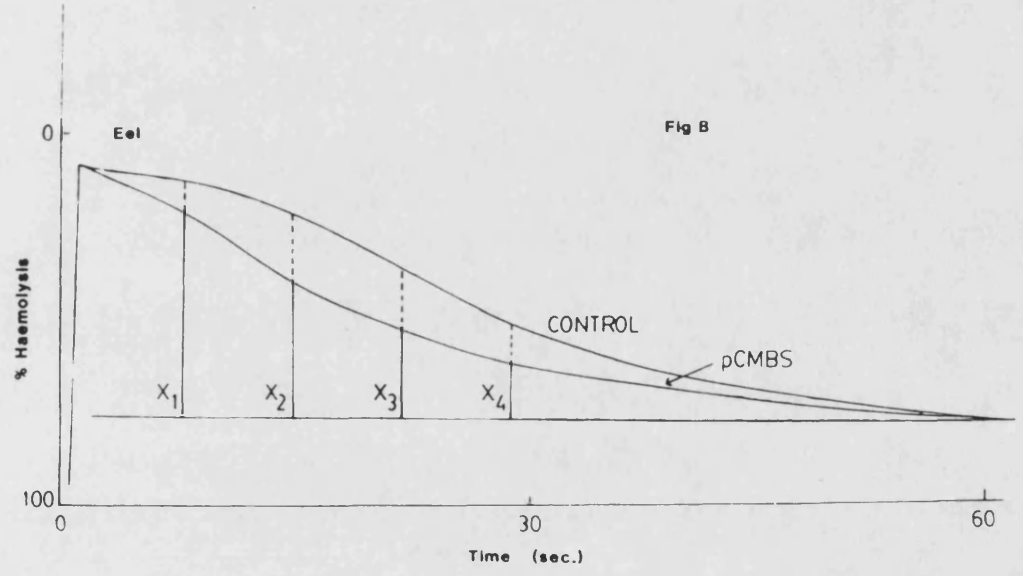
Procedure:

50 μl of erythrocyte suspension at 10-15% haematocrit were pipetted into a 4 ml cuvette in a temperature controlled carriage of a linear read out grating spectrophotometer (CECIL instrument CE 373). The tonicity of the suspension was disturbed by the rapid injection of 3 ml of hypotonic solution. 65-70 mM and 45-50 mM NaCl solution were used in human and eel experiments respectively to produce 75% haemolysis in one minute. The resulting haemolysis curve was recorded by an Omniscribe recorder connected to the spectrophotometer. Experimental runs were conducted at least twice on each blood sample and the means used in data analysis.

Data Analysis:

The haemolysis curve can be analysed by plotting the logarithm of signal magnitude ($x_1 \longrightarrow x_4$) against the time (Fig A & B). The individual points were obtained by measuring the vertical distance of each trace from its corresponding asymptote for a series of times. Distance values obtained for a given time were then logged and





Figs A & B Data taken from typical trace for human and eel subjected to haemolysing solutions.

plotted against the time. The rate of haemolysis can be obtained from the slope of the least squares fit for these points. This manner of plotting tends to minimize unessential difference while leaving the fundamental relations clearly visible. It is the rates rather than the absolute values of the times of haemolysis that are of particular significance.

The temperature relationship can be obtained from the Arrhenius plot of the logarithm of haemolysis rate against the reciprocal of absolute temperature T (K^{-1}). From the slopes of the different lines obtained, by the least square method, the activation energy (E_a) for each set of conditions could be calculated and expressed in Joules mol^{-1} (see equation 2 page 10).

2.2.2.2 The Nuclear Magnetic Resonance Technique: Spin-Spin Relaxation Time (T_2) Measurement (Diffusional permeability)

The NMR relaxation time method is an accurate, sensitive technique for measuring the time course for diffusional water movement across the red cell membrane. The basic principle of the technique is that, water protons placed in a static magnetic field of 90 M Hz can absorb energy from a radio-frequency magnetic field. The decay of the coherent form of this absorbed energy can be measured by the standard Tranverse relaxation time T_2 Technique, (Conlon and Outhred , 1972).

After applying a brief intense radio frequency pulse to a blood sample in a suitable magnetic field, the resultant coherence of the proton spins temporarily labels the blood magnetically. The spontaneous decay time, T_2, of the label for water both inside and outside the red cell is about 140 milliseconds. By incorporating an impermeable paramagnetic ion (eg Mn^{++}) in the plasma the relaxation time for extracellular protons can be made very short ie; $<$ 10 ms and in this way the label decay time within the cells would be dominated by the proton exchange process from cell to plasma. Water molecules leaving the cells would lose their magnetic label soon after contacting the plasma containing Mn^{++} and those entering the cells would all be unlabelled.

Experimental procedure:

The red blood cells were used within 4-8 hours of sampling. The sample was made by mixing 1 ml of blood with 0.5 ml 40 mM $MnCl_2$ solution in 110 mM NaCl.

The NMR measurements were performed with a Jeol Fx 90 NMR spectrometer at 90 MHz. The spin-spin relaxation time was measured by the CPMG method (Carr-Purcell-Meiboom-Gill), (Abraham and Loftus, 1981). The specimen temperature in the spectrometer was controlled to $\pm$ 0.2°C for temperatures higher than room temperature. At lower temperatures a stream of liquid nitrogen was used to control the temperature to $\pm$ 0.5°C or better. In both cases the actual temperature in the blood sample was measured using a

thermocouple.

In order to achieve the optimum mixing of the Mn^{++} doping solution with the blood, Morariu and Benga (1977) recommend two ways for sample preparation.

1) The doping solution may be added to the blood in the NMR tube with gentle rotation in the almost horizontal position for about 10-15 times. This preparation used in the present work.

2) The blood can be pipetted on to the bottom of a 150 ml pyrex flask and the doping solution spread over the blood followed by rotation of the flask several times. The sample is then transferred to the NMR Tube.

Conlon and Outhred (1972) reported that prolonged shaking leads to a disappearance of the two component relaxation behaviour, but Morariu and Benga (1977) were not able to detect such effect and stated that the only case of disappearance of longer relaxation component was in a sample of transfusion blood that had been stored for about three months at 4°C.

Data Analysis:

The spin-spin relaxation time was evaluated from the logarithmic plot of the nuclear spin echo as a function of 2τ, where τ is the time interval between the radiofrequency pulses (Abraham and Loftus, 1981) when the

kinetics of the system is characterised by a single relaxation time, the plot is a straight line and the relaxation time is the reciprocal of the slope. For a system characterised by two relaxation times, as for blood doped with Mn^{2+}, the plot consists of two lines as shown in figure (C). Initially, the relaxation is dominated by rapid decay of the plasma label. After a few milliseconds the plasma label has effectively disappeared, thereafter only the intracellular label remains and this decays more slowly, principally by water diffusion into the plasma. Figure (D) shows the decay in time of the water proton spin echo in a blood sample doped with $MnCl_2$. Component b results from the rapid decay of spin echo in plasma.

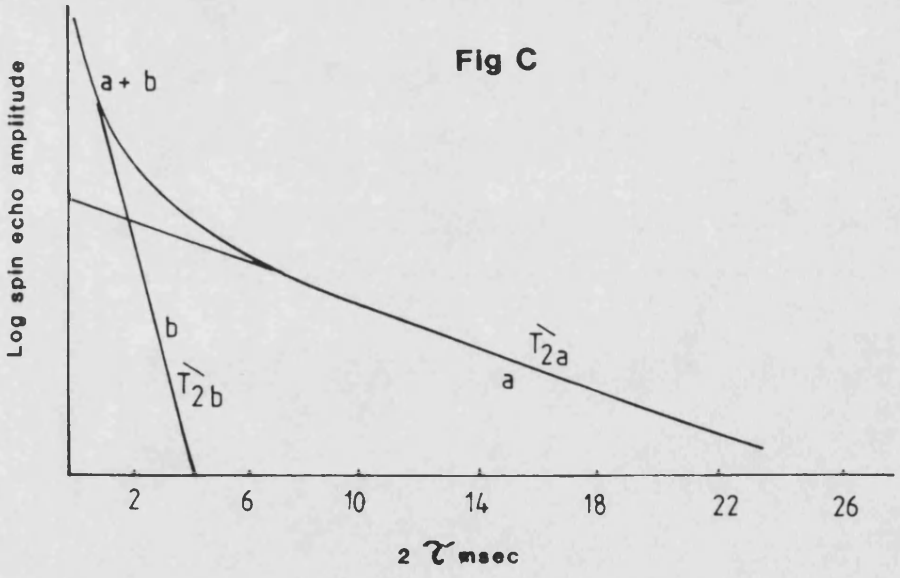


Fig C

Component 'a' results from the slower decay of spin echo in red blood cells. Component 'a + b' results from the rapid decay of the whole sample.

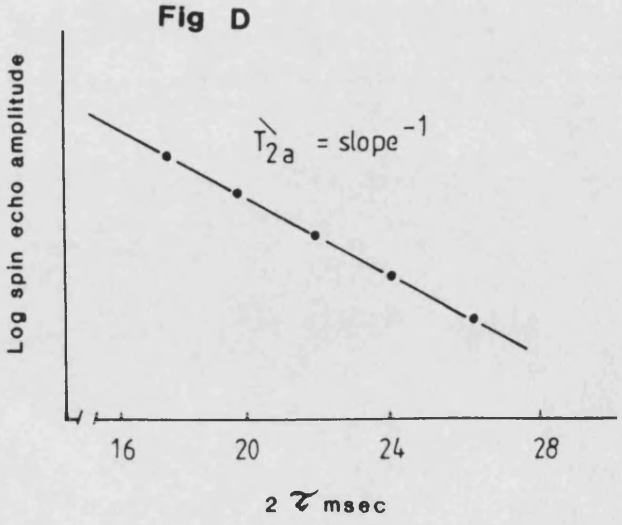
The observed relaxation time is related to the water diffusion exchange time by the equation:

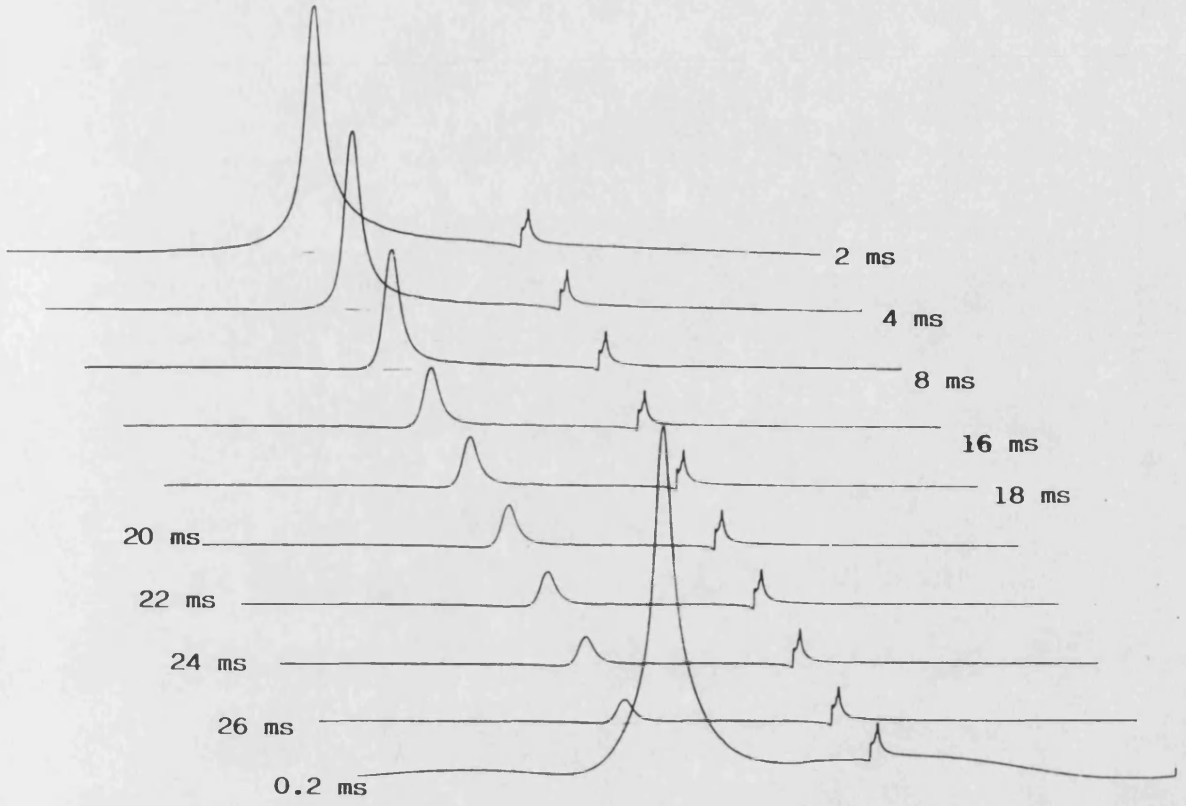
$$T'_{2a}{}^{-1} = T_a{}^{-1} + T_{2a}{}^{-1} \qquad (3)$$

(Conlon and Outhred (1972 & 1978) and Morariu and Benga (1977)) where T'_{2a} is the observed relaxation time of the slow decay component; T_{2a} the apparent decay time in isolated red blood cells, T_a is the apparent exchange time which is the quantity of interest.

The relaxation time T'_{2a} was measured from the spin echo attenuation in the region between $16 - 2\tau - 26$ ms which corresponds to the slowest decay fraction. The logarithmic plot of the nuclear spin echo as a function of time intervals is linear in this region (figure (C)).

Fig D





A typical trace of NMR spectrum for water relaxation time T_2.

2.2.2.3 Determination of Packed Cell Volume (Haematocrit)

The packed cell volume measurement was made at room temperature by the standard microhaematocrit technique (Dacie and Lewis, 1968). Well mixed, heparinised blood was taken up into a microhaematocrit-tube (75mm long, 1.1-1.2 mm internal diameter) for a length of about 50 mm, at least two tubes being prepared from each sample. One end was sealed with critoseal and the tubes centrifuged in a microhaematocrit centrifuge at 10,000 rpm for 15 min. The haematocrit was determined with the aid of a microhaematocrit reader and the values expressed as volume of cells per 100 cm^3.

2.2.2.4 Determination of the Osmolalities:

Osmolalities of all solutions were measured in 50 μ samples of fluid using a Roebling freezing point depression micro-osmometer model MOD 200, calibrated at 290 mosm Kg^{-1}.

2.2.2.5 Determination of pH:

The pH of all solutions used were adjusted by a PTI-15 Digital PH meter, calibrated at PH 7, 5 and 8.

2.3 RESULT

The temperature dependence of the rate of haemolysis due to osmotic water movement across the erythrocyte membrane is described in terms of an Arrhenius plot and expressed as an activation energy E_a. The rate of haemolysis was measured in Anguilla anguilla and human erythrocytes together with the effect of pHMB on the E_a. In all Arrhenius plots the S.Ds have been omitted for clarity but their ranges have been quoted in the legends.

2.3.1 Eel Erythrocytes

Table III and Fig I show the Arrhenius plot and computed activation energies for haemolysis rates for unwashed eel red cells at pH 7.4 brought about by exposure to hypotonic buffered solution. The data show a discontinuity at around 27.5°C with two apparent activation energies 24.9 KJ mol^{-1} (r^2 = 0.989) below the discontinuity (at the lower temperature range) and 3.6 KJ mol^{-1} (r^2 = 0.986) above the discontinuity (the higher temperange). The mean overall activation energy is 21.9 KJ mol^{-1} (r^2 = 0.97) for the whole range of temperature (7-37°C). The effect of 1 mM pHMB on the same system is also represented in table III and fig I and show a one line fit without discontinuity through the studied temperature range and a lowered energy of activation of 9.1 KJ mol^{-1} (r^2 = 0.955).

Table III and fig II illustrate the Arrhenius plots for the haemolysis rate due to osmotic water flow across washed untreated and pHMB treated eel erythrocytes at pH 7.4. In the temperature range studied (7-40°C) no deviation from linearity was observed. The activation energies for haemolysis rates of untreated and pHMB treated red cells are 15.6 KJ mol^{-1} (r^2 = 0.997) and 10.8 KJ mol^{-1} (r^2 = 0.970) respectively.

Table (III) Activation energies characterizing the temperature dependence of osmotic haemolysis of eel erythrocytes at pH 7.4

Sample	overall E_a (KJ mol^{-1})	r^2 (n)	t_{disc} °C	$E_a < t_{disc}$ (KJ mol^{-1})	r^2 (n)	$E_a > t_{disc}$ (KJ mol^{-1})	r^2 (n)
unwashed untreated	21.9	0.970 (11)	27	24.9	0.989 (8)	3.6	0.986 (4)
unwashed + pHMB	9.1	0.95 (13)	None	-		-	
washed untreated	15.6	0.997 (10)	None	-		-	
washed + pHMB	10.8	0.970 (10)	None	-		-	

E_a is the activation energies in KJ mol^{-1}

t_{disc} is the temperature of discontinuity of the Arrhenius plot.

$E_a < t_{disc}$ is the activation energy at temperatures lower than t_{disc}.

$E_a > t_{disc}$ is the activation energy at temperatures higher than t_{disc}.

r^2 is the correlation coefficient.

n is the number of individual points.

Fig I and II

Arrhenius plot of haemolysis rate across eel erythrocyte membranes at pH 7.4 of untreated and pHMB treated cells. SD of mean of all points between $\pm$ 0.016 to $\pm$ 0.039.

Fig I unwashed erythrocytes

Fig II washed erythrocytes

Abbreviations used in Arrhenius plots only:

E_a = Activation energy in KJ mol^{-1}

t_{disc} = Temperature of discontinuity

$E_a < t_{disc}$ = Activation energy at temperature lower than t_{disc}

$E_a > t_{disc}$ = Activation energy at temperature higher than t_{disc}

r^2 = Correlation coefficient

Fig I

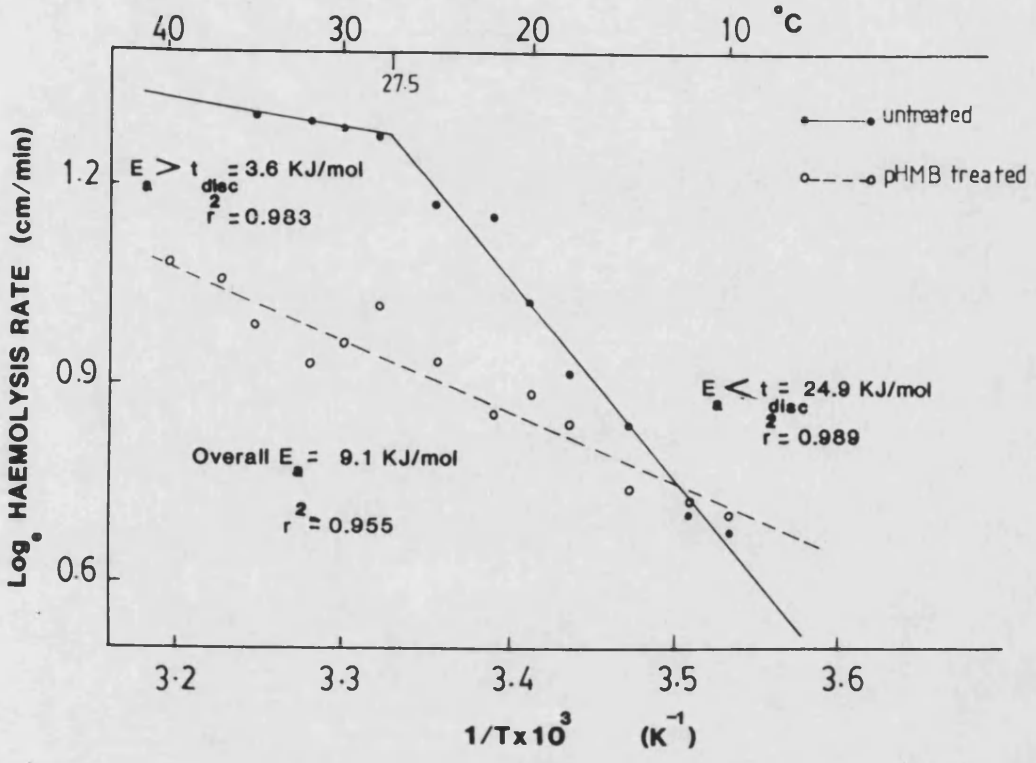
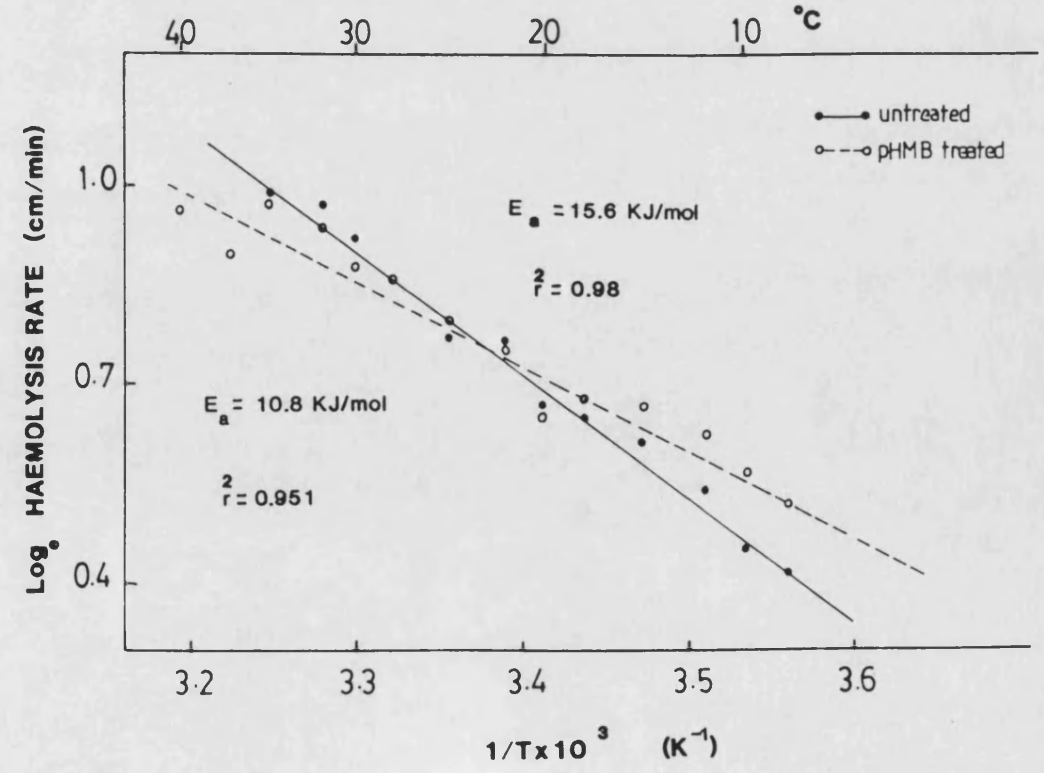

40
30
20
10
°C
27.5
untreated
pHMB treated
$E_a > t_{disc}$ = 3.6 KJ/mol
r^2 = 0.983
$E_a < t_{disc}$ = 24.9 KJ/mol
r^2 = 0.989
Overall E_a = 9.1 KJ/mol
r^2 = 0.955
Log$_e$ HAEMOLYSIS RATE (cm/min)
1.2
0.9
0.6
3·2
3·3
3·4
3·5
3·6
1/Tx10^3 (K^{-1})

Fig II


40
30
20
10
°C
untreated
pHMB treated
E_a = 15.6 KJ/mol
r^2 = 0.98
E_a = 10.8 KJ/mol
r^2 = 0.951
Log$_e$ HAEMOLYSIS RATE (cm/min)
1.0
0.7
0.4
3·2
3·3
3·4
3·5
3·6
1/Tx10^3 (K^{-1})

2.3.2 Human Erythrocytes

The temperature dependence of the rate of osmotic haemolysis due to the inward osmotic water movement across the human erythrocyte membrane was examined at 3 different pHs: 7.4, 6.8 and 6.4. Only selected Arrhenius plots havebeen presented, most data having been tabulated.

pH 7.4

It is evident by inspection of the data in table IV for unwashed,untreated erythrocytes and from the correlation coefficients (r^2), that a two line fit is slightly better but very close to a one line fit. The two lines were obtained by varying the junction between them so as to maximise the correlation coefficient (r^2) of each. The phase transition between the two lines, if it exists, is around 22.8°C (t_{disc}). The mean overall activation energy is 11.6 KJ mol^{-1} (r^2 = 0.976) with the two lines having activation energies of 7.5 KJ mol^{-1} (r^2 = 0.962) at the lower temperature range and 15 KJ mol^{-1} (r^2 = 0.992) at higher temperature range. These results show that osmotic water flow changes markedly with temperature at higher temperature range ($>$ t_{disc}) whereas at the lower temperature range ($<$ t_{disc}) there is little change.

The effect of pHMB (2 mM) on the same experimental system is also presented in table IV. The data show a weak phase transition at t_{disc} = 20°C with two values of the apparent

activation energies of 9.1 KJ mol^{-1} (r^2 = 0.99) and 18.4 KJ mol^{-1} (r^2 = 0.98) respectively below and above the temperature of the discontinuity. The mean overall activation energy is 14.5 KJ mol^{-1} (r^2 = 0.974). The data show little difference between the E_a for unwashed pHMB-treated and untreated cells and could probably be attributed to the binding of pHMB with the extracellular proteins present in the plasma and their non-availability to bind with the membrane.

The plotted Arrhenius data in fig III for washed human red cells suspended in saline solution at pH 7.4 shows a discontinuity around 19°C with two apparent activation energies above and below the phase transition of 11.1 KJ mol^{-1} (r^2 = 0.993) and 3.8 KJ mol^{-1} (r^2 =0.972) respectively, the mean overall activation energy being 8.4 KJ mol^{-1} (r^2 = 0.974).

The results of temperature dependent haemolysis in human erythrocytes treated with pHMB at pH 7.4 are plotted in figure IV and table IV. In the temperature range studied (6-44°C), a deviation from linearity was observed around 35-37°C. Energies of activation for water transport across the membrane are 18.1 KJ mol^{-1} (r^2 = 0.989) from 37°C to 6°C and 4.8 KJ mol^{-1} r^2 = 0.92 above 37°C. The overall activation energy (6-44°C) is 16.4 KJ mol^{-1} (r^2 = 0.985).

It is also evident from table IV that the washed erythrocytes showed lower activation energies than unwashed

red cells. Treatment of washed erythrocyte with 2 mM pHMB showed a 94% increase in the activation energy over the value for control cells.

Table IV: Parameters characterizing the osmotic haemolysis rate due to osmotic water permeation through human erythrocyte membranes at pH 7.4*

Sample	overall E_a (KJ mol^{-1})	r^2 (n)	t_{disc} °C	$E_a < t_{disc}$ (KJ mol^{-1})	r^2 (n)	$E_a > t_{disc}$ (KJ mol^{-1})	r^2 (n)
unwashed untreated	11.6	0.976 (14)	22	7.5	0.962 (7)	15.00	0.992 (10)
unwashed + pHMB	14.5	0.974 (14)	20	9.1	0.99 (6)	18.40	0.980 (9)
washed untreated	8.4	0.974 (14)	19	3.8	0.973 (6)	11.1	0.993 (11)
washed + pHMB	16.4	0.985 (14)	35-37	18.1	0.989 (12)	4.8	0.92 (4)

***The notations are the same as in table III.**

pH 6.8

Table V, shows the temperature dependent water permeation of unwashed untreated human erythrocytes at pH 6.8. By inspection of the data and the correlation coefficients (r^2), it is again evident that a two line fit is slightly better than a one line fit. The break is at 26.5°C (t_{disc}). The mean overall activation energy is 11.4 KJ

Fig III and IV

Arrhenius plot of haemolysis rate of washed human erythrocyte membranes at pH 7.4 SD ($\pm$ 0.014 to $\pm$ 0.045)

Fig III untreated

Fig IV pHMB treated

Fig III

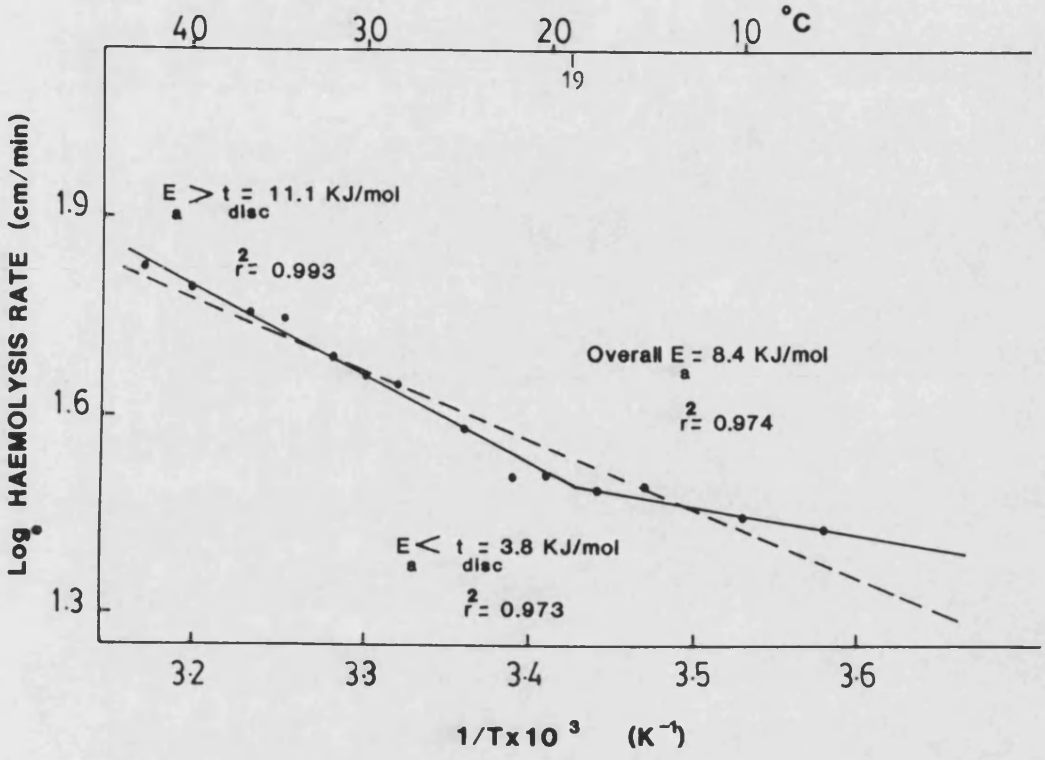
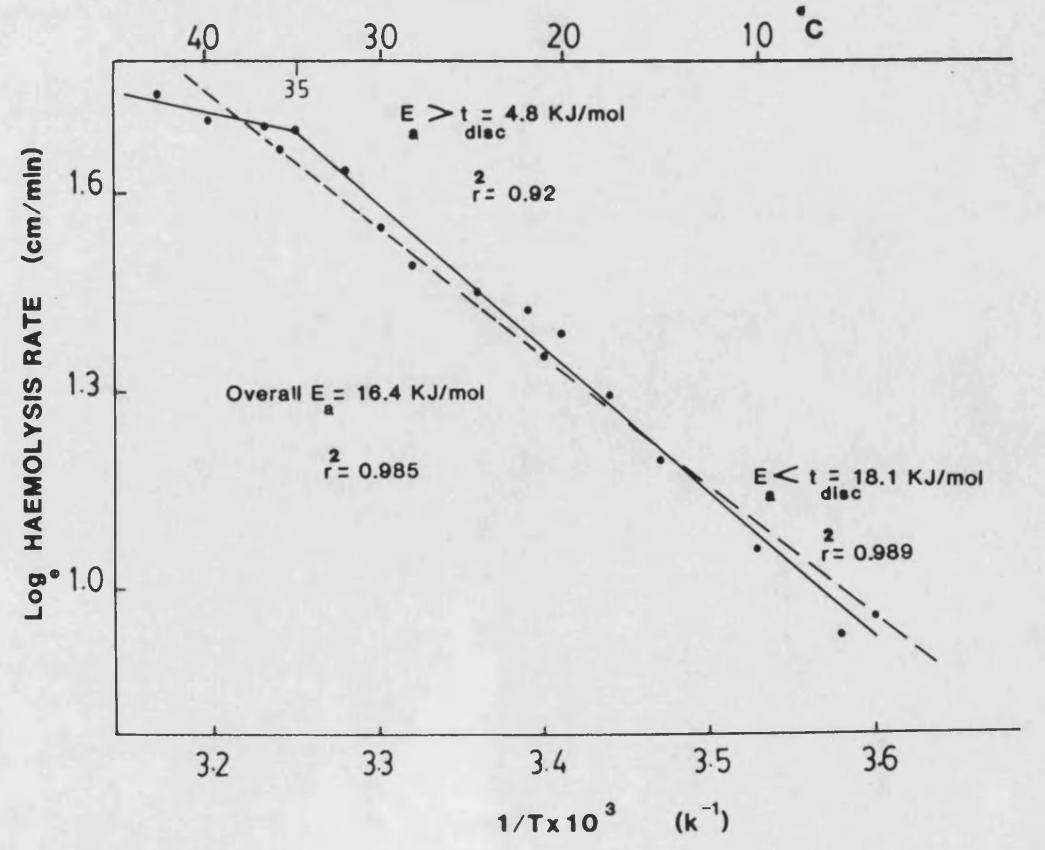


Fig IV



mol^{-1} (r^2 = 0.979), and two line means are 16.5 KJ mol^{-1} (r^2 = 0.99) at higher temperatures and 8.2 KJ mol^{-1} r^2 = 0.99 at lower temperatures.

The effect of 2 mM pHMB on the same system shows a 28% increase in the mean overall activation energy to 14.6 KJ mol^{-1} (r^2 = 0.922). A phase transition is seen at a similar temperature as untreated cells, ie 26°C and the two activation energies calculated below and above the discontinuity are 5.21 KJ mol^{-1} (r^2 = 0.991) and 27.4 KJ mol^{-1} (r^2 = 0.983) respectively.

The temperature dependence of haemolysis rate for washed untreated erythrocytes suspended in saline solution at pH 6.8 is illustrated in table V. The data show an obvious phase transition at 27°C (t_{disc}) and two apparent activation energies below and above the discontinuity of 4.2 KJ mol^{-1} (r^2 = 0.998) and 20.5 KJ mol^{-1} (r^2 = 0.995) respectively. A one line fit is unlikely because of its low correlation coefficient (r^2 = 0.91) compared to the two line fit. The mean overall activation energy is 10.3 KJ mol^{-1}.

The results from the same system treated with 2 mM pHMB are shown also in table V. In the temperature range studied (6-44°C) no deviation from linearity was observed, ie the phase transition is removed. Treatment with 2mM pHMB produced an increase in the activation energy to 29 KJ mol^{-1} (r^2 = 0.996) amounting to 180% increase on the value

measured with the washed control cells at pH 6.8.

Table V: Parameters characterising osmotic haemolysis rate due to osmotic water permeation through human erythrocyte membranes at pH 6.8.*

Sample	overall E_a (KJ mol^{-1})	r^2 (n)	t_{disc} °C	$E_a < t_{disc}$ (KJ mol^{-1})	r^2 (n)	$E_a > t_{disc}$ (KJ mol^{-1})	r^2 (n)
unwashed untreated	11.4	0.979 (13)	26.5	8.2	0.991 (6)	16.5	0.99 (8)
unwashed + pHMB	14.6	0.922 (13)	26	5.2	0.991 (7)	27.4	0.983 (8)
washed untreated	10.3	0.91 (13)	27	4.2	0.998 (7)	20.50	0.965 (8)
washed + pHMB	29	0.996 (13)	-	-		-	

***The notations are the same as table III.**

pH 6.4

The temperature dependence of the rate of haemolysis in human unwashed untreated erythrocyte membranes at pH 6.4 is illustrated in table VI. The phase transition in the Arrhenius plot is much less evident than at pH 7.4 and 6.8,

however, a t_{disc} (temperature of discontinuity) of 22°C could be estimated by intersecting the best two lines with highest correlation coefficients. The mean overall activation energy is 11.50 KJ mol^{-1} (r^2 = 0.983). The two activation energies, calculated from the best two lines, are 14.1 KJ mol^{-1} (r^2 = 0.973) and 9.4 KJ mol^{-1} (r^2 = 0.97) below and above the estimated discontinuity respectively.

The effect of 2 mM pHMB on the same system shows no phase transition throughout the studied temperature range (6-44°C) and an energy of activation of 15.2 KJ mol^{-1} (r^2 = 0.993), table VI.

The haemolysis rates of washed cells at pH 6.4 were examined and the activation energies were presented in table VI. The data show that a two line fit is slightly better but very close to a one line fit. A phase transition between the two lines is at 20°C (t_{disc}). The mean overall activation energy is 12.4 KJ mol^{-1} (r^2 = 0.98) and energies of activation below and above the discontinuity are 8.6 KJ mol^{-1} (r^2 = 0.995) and 13.5 KJ mol^{-1} (r^2 = 0.991) respectively.

The addition of 2 mM pHMB on the same system at pH 6.4 is shown in table VI. The data show that a two line fit (with discontinuity (t_{disc}) = 23.5°C) is very close to a one line fit and there is an increase in the mean overall activation energy to 19.2 KJ mol^{-1} (r^2 = 0.975). The major effect of pHMB is at lower temperatures with an increase in energy of

activation to 27.1 KJ mol^{-1} (r^2 = 0.995) but with little change above the discontinuity (E_a = 12.3 KJ mol^{-1} (r^2 = 0.965).

Table VI: Parameter characterizing osmotic haemolysis rate due to osmotic water permeation through human erythrocyte membranes at pH 6.4*

Sample	overall E_a (KJ mol^{-1})	r^2 (n)	t_{disc} °C	$E_a < t_{disc}$ (KJ mol^{-1})	r^2 (n)	$E_a > t_{disc}$ (KJ mol^{-1})	r^2 (n)
unwashed untreated	11.50	0.983 (14)	22 calculated	14.1	0.973 (7)	9.4	0.970 (10)
unwashed + pHMB	15.2	0.993 (13)	-	-	-	-	-
washed untreated	12.4	0.980 (14)	20	8.6	0.995 (6)	13.5	0.991 (10)
washed + pHMB	19.2	0.975 (14)	23.4	27.06	0.995 (7)	12.30	0.965

*The notations are the same as in table III.

2.3.3 Temperature dependence of water diffusion exchange time of human unwashed erythrocyte membranes

The results from temperature dependent measurements of water diffusion exchange time (T_2) for human unwashed erythrocytes at pH 7.4 as measured by the NMR technique are plotted in fig V. In the temperature range (10 - 45°C) investigated no phase transition was observed. The energy of activation for water diffusion exchange time is 29.80 KJ mol^{-1} (r^2 = 0.997). The values of diffusional exchange time (T_2) at various temperatures are listed in table VII.

Table VII: Water diffusion exchange time through human unwashed erythrocyte membrane for temperature range in between 10–45°C at pH 7.4 measured by the NMR technique

Temp °C	10	15	20	25	30	35	37	40	45
T_2 (m sec)	18.45	16.29	13.15	10.89	8.89	7.18	6.367	5.62	4.90

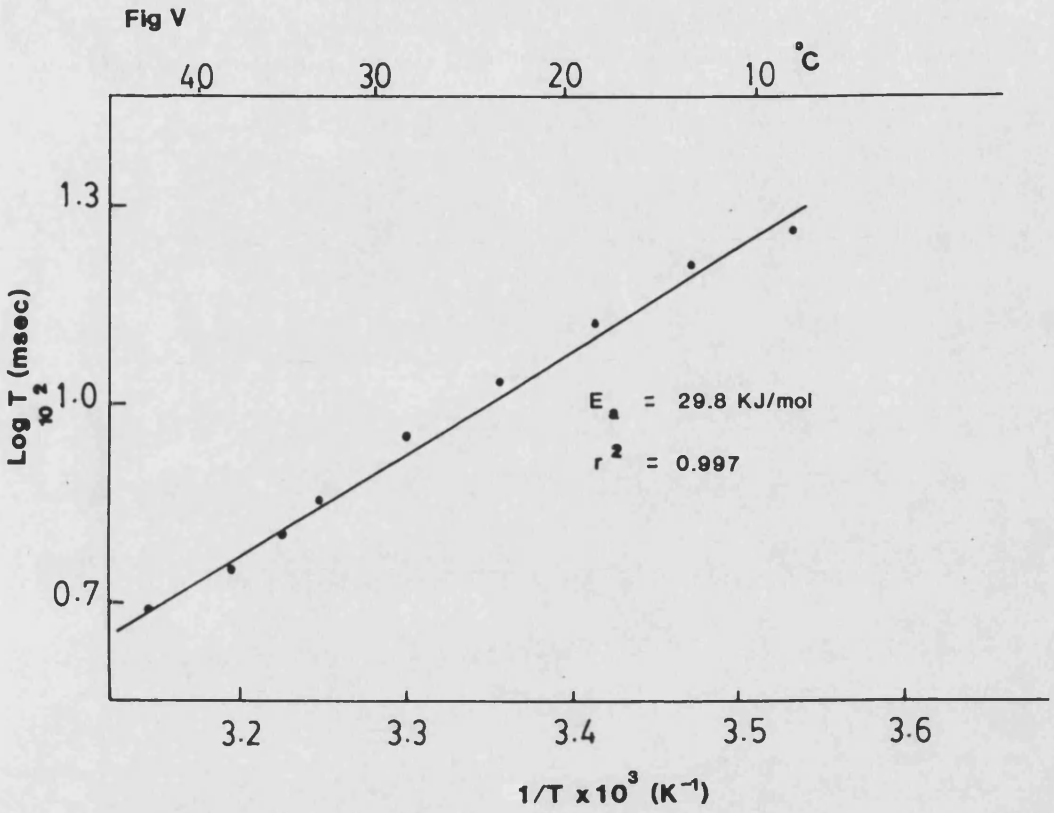


Fig V Arrhenius plot of water exchange time (T_2) of diffusional water transport across human erythrocyte membranes at pH 7.4.

2.3.4 Effect of pCMBS on the rate of water exchange time ($1/T_e$) in human and eel erythrocyte membranes

Table VIII: The effect of pCMBS on $1/T_e$ in human and eel red blood cells at 15°C pH 7.4

CELL	$1/T_e$	T_e m sec	% of inhibition acceleration
Human erythrocytes	0.062	16.3	18%
Human erythrocytes + pCMBS	0.050	20	inhibition
Eel erythrocytes	0.043	23.1	32%
Eel erythrocytes + pCMBS	0.057	17.5	acceleration

As shown in table VIII treament with 2 mM pCMBS resulted in an increase in the water exchange time (T_e) and hence an 18% inhibition of the rate of water diffusion across human erythrocyte membranes at pH 7.4 and 15°C. In eel erythrocytes, treatment with 1 mM pCMBS showed a decreased water exchange time (T_e) which resulted in a 32% acceleration of the rate of water diffusion across the membranes at pH 7.4 and 15°C.

2.4 DISCUSSION

For human cells under an osmotic stress, the final percentage haemolysis achieved is far greater at 15°C than at 37°C (table IX p 58). This apparent anomally is in agreement with the earlier work of Jacobs and Parpart (1931). In contrast, eel cells under osmotic stress show final percentages of haemolysis that are temperature independent.

There has been two alternative explanations voiced for the human data; one, which relates to the effect of temperature on intracellular phenomena (eg prelytic loss of K^+) and the other on the membrane. The work of Aloni et al (1977) appears to conclusively identify the membrane as the major effector in these observations. The fact that eel cells (an ectotherm) show a temperature independent haemolysis suggests that it may be due to the level of saturated to unsaturated fatty acids present in the membrane that determines the response. Temperature dependent phase transition, often attributed to membrane lipid components, may also be strong candidates for these observations and osmotic and diffusional data can help to identify these particular sites for water transport.

2.4.1 Thermal Transition in Arrhenius Plots of Permeability:

Any break in the linear relationship of an Arrhenius plot may be attributed to a phase transition in the lipid (mobile $\rightleftharpoons$ crystaline state) or protein (sol $\rightleftharpoons$ gel)

components of the membrane, each of which will affect the passage of water molecules either by diffusion through the lipid phase or by diffusion and bulk flow through aqueous channels in the protein phase. The presence or absence of such transitions is still in dispute in the literature and evidence from this work, although failing to conclusively prove their presence, suggests that components within the suspending media influence their experimental observation.

The temperature dependence of water permeation through erythrocyte membranes investigated by Vieira et al (1970), Morariu and Benga (1977), and Pitterich and Lawaczeck (1985) have all reported linear Arrhenius plots with no discontinuities. Several other investigators, however, have discovered such discontinuities using the following erythrocyte membrane parameters: viscosity and micro-viscosity (Zimmer and Schirmer (1974), Feinstein, et al (1975)), osmotic fragility (Aloni, et al (1977)), transport of chloride and bromine (Brahm and Wieth (1977)), exchange transport of glucose (Lacko, et al, (1973)) and lithium efflux (Levy et al (1982). All these phenomena have been discussed in terms of a phase transition in erythrocyte membrane occurring between 19-25°C.

The data from this study, collected together in table VIII show that where there are discontinuities in the Arrhenius plots for osmotic haemolysis, they are evident in the case of washed rather than unwashed human cells. Their plots

Table IX : Summary of Erythrocytes water transport activation energies in KJ mol^{-1}

Sample	pH	overall E_a (KJ mol^{-1})	r^2 (n)	t_{disc} °C	$E_a < t_{disc}$ (KJ mol^{-1})	r^2 (n)	$E_a > t_{disc}$ (KJ mol^{-1})	r^2 (n)
unwashed	7.4	21.9	0.970 (11)	27	24.9	0.989 (8)	3.6	0.986 (4)
+ pHMB	"	9.13	0.955 (13)					
washed	"	15.6	0.997 (10)					
+ pHMB	"	10.8	0.970 (10)					
unwashed	7.4	11.6	0.976 (14)	22.8	7.5	0.962 (7)	15.00	0.992 (10)
+ pHMB	"	14.5	0.974 (12)	20	9.1	0.99 (6)	18.40	0.98 (9)
washed	"	8.4	0.974 (14)	19	3.8	0.973 (6)	11.1	0.993 (11)
+ pHMB	"	16.4	0.985 (14)	35-37	18.1	0.989 (12)	4.8	0.92 (4)
unwashed	6.8	11.4	0.979 (13)	26.5	8.2	0.991 (6)	16.5	0.99 (8)
+ pHMB	"	14.6	0.922 (13)	26	5.2	0.991 (7)	27.4	0.983 (8)
washed	"	10.3	0.91 (13)	27	4.2	0.988 (7)	20.5	0.965 (8)
+ pHMB	"	29	0.996 (13)					
unwashed	6.4	11.5	0.983 (14)	22 calculated	14.1	0.973 (7)	9.4	0.970 (10)
+ pHMB	"	15.2	0.993 (14)					
washed	"	12.4	0.980 (14)	20	8.5	0.995 (6)	13.5	0.991 (10)
+ pHMB	"	19.2	0.975 (14)	23.4	27.1	0.995 (7)	12.30	0.965 (9)

are relatively linear with high correlation coefficients (r^2 = 0.91 - 0.97) at pHs 7.4 and 6.8 and completely linear (r^2 = 0.98) at pH 6.4.

The Arrhenius plots for eel erythrocytes again show no deviation from linearity for washed cells. There is a break for unwashed cells at 27°C and, being a physiologically abnormal temperature, may be due to considerable membrane change.

In the present study any discontinuities found, occurred within the range of 19-27°C with two linear regions for lower and higher temperatures (table VIII). These data are in agreement with the discontinuity range of 20-30°C recorded previously by Conlon and Outhred (1978) and Mororiu et al, (1981) at pHs 7.4 and 7.0 for isolated red cells. At pH 6.4, there is an agreement between the present results and that of Morariu et al (1981) in that the break is much less evident than at pH 7.4 with a value estimated at 22°C.

Treatment of eel erythrocytes with the cross-linking sulphydryl compound, pHMB produces complete linearity through the studied temperature range in both washed and unwashed cells. Similarly, in human erythrocytes, treatment with pHMB shows almost complete linearity at all pHs in both washed and unwashed cells.

The temperature dependence of water exchange time, T_2, as measured by NMR for unwashed human erythrocytes at pH 7.4

(13 mM Mn^{2+}), again showed no break in the Arrhenius plot and is in agreement with the data of Pirkle et al (1979). Although, Morariu et al (1985) demonstrated breaks in apparent water exchange times for washed erythrocytes doped with high concentrations of manganese (30 mM), they found no such discontinuities at low manganese concentrations (5-8 mM) and suggested that any transition observed by the NMR method was due to the entry of the paramagnetic Mn^{2+} ions into the cells. In unwashed blood, no discontinuities were observed at high or low Mn^{2+} concentrations, an observation suggesting that extracellular components may influence this observation. This absence of the thermal transition in Arrhenius plots was also recently observed in the work of Pitterich and Lawaczeck (1985) at temperature range between 5 - 35°C of water and proton permeation across membranes for human and bovine ghosts.

In summary, the present results do not fully establish the definite presence of a phase transition for water permeation through either eel or human red cells as measured by either osmotic haemolysis or by the NMR technique. The Arrhenius diagrams revealed a linearity for unwashed cells and a weak change (thermal transition) at 19-22°C with two linear regions with correlation coefficients very close to that of a single line fit (table IX). Because of this, the observed transition must be considered to be weak. The appearance of a thermal transition was always shown to be associated with isolated erythrocytes out of their plasma suggesting the importance

of a plasma constituent for providing factor(s) influencing membrane stability and structure. In this connection, it is possible that a plasma constituent containing -SH groups (eg albumin) would be critical, as the addition of the specific sulphydryl reagent, pHMB, to the washed erythrocyte showing weak transitions, cross-linked the membrane -SH groups and lead to the disappearance of the phase transition.

It is of course conceivable that, the appearance of a thermal transition is associated with and could be due to a methodological artifact in the determination of water permeability, such has been observed during the NMR technique with the entry of paramagnetic Mn^{2+} ions into cells (Morariu et al, 1985).

2.4.2 Activation Energy (E_a) for Water Permeability

The osmotic transport of water across unwashed eel erythrocytes, measured as rates of haemolysis has a mean overall activation energy (E_a) of 21.9 KJ mol^{-1}, a value similar to those for self diffusion and bulk water viscosity; 19.3 - 20.1 KJ mol^{-1} (Wang et al (1953), Wang (1965), Mills (1973) and House (1974)). The activation energy of 15.57 KJ mol^{-1} for washed eel erythrocytes is identical to that for the dog erythrocyte as measured by Vieira et al (1970). These authors found that viscous flow of water takes place across the dog red cell membrane

when an osmotic pressure gradient is applied. With such similar E_a values , it may be proposed that the osmotic flow of water across washed eel membranes could also be viscous in character although it should be pointed out that the value is lower than the 20 KJ mol^{-1} for viscous flow.

The activation energy for osmotic water transport through human erythrocyte membranes was even lower than that for water transport in free solution being about 11.5 KJ mol^{-1} for unwashed erythrocytes at all pHs measured. These values for human cells are in accord with those of the 13.8 KJ mol^{-1} reported by Vieira et al (1970) and Naccache and Sha'afi data at pH 7.4 and lends credance to the acceptable methodological approach in this work.

Little data has appeared in the literature for washed human cells and this present investigation reveals that their overall activation energy is particularly low at 8.4 KJ mol^{-1} at pH 7.4 and 6.8, compared to unwashed cells, although at pH 6.4 the values for washed and unwashed cells are very close (12.4 KJ mol^{-1} and 11.50 KJ mol^{-1} respectively). A similar observation was made for eel erythrocytes. Like the phase transition observation, this difference may be attributed to the loss of a plasma fraction that influences membrane interaction with water movement. The identity of the fraction(s) was not discovered but preliminary data showed that neither albumin, Ca^{++} nor Mg^{++} ions were involved.

The human data reveal that in those cases that exhibit a weak phase transition (table IX) the higher activation energies (20 - 11 KJ mol^{-1}) were observed at higher temperatures above the discontinuity and the lower activation energies (8.5 - 3.78 KJ mol^{-1}) were recorded below the discontinuity. In the case of eel unwashed cells, the reverse is the case, the higher value for E_a of 24.9 KJ mol^{-1} was recorded at the normal physiological range of the animal while low values 3.6 KJ mol^{-1} was found about 27°C. This variability in values is not uncommon in the literature, for Morariu et al (1981) showed an even more pronounced change in activation energy for water exchange time (T_2) in washed human erythrocyte, from 6 KJ mol^{-1} below 26°C to 24 KJ mol^{-1} above that temperature, values that are in close accord with those in this study. A recent NMR study (Morariu et al, 1985) showed a particularly interesting phenomena of washed red blood cells collected from infants (less than 48hrs old) in which the Arrhenius plot consisted of two different linear regions with slopes of opposite signs recorded at - 2.1 KJ mol^{-1} for low temperature below 13°C and 3.9 KJ mol^{-1} for higher temperatures. Strangely, Morariu considered that these low positive or negative values can have no meaning for water diffusion. Such values for activation energies and in particular those with either positive Arrhenius slopes or values significantly lower than that for self-diffusion of water, raise important issues as to the mechanistic explanation for them.

Vieira et al (1970) interpreted the values for activation energies on the bais that water transport could be described by movement through equivalent pores in the membrane, and that low values below 20 KJ mol^{-1} suggested some water slippage at the water membrane interface that assisted flow in some way. Brahm (1982) commented that if such slippage does explain the low activation energy of water movement, then the phenomenon itself, as a part of the transport process, must be almost insensitive to temperature.

Values for apparent activation energies for water diffusion are higher than those for self diffusion. This confirms literature values for human red cells measured both using tritiated water (Vieira et al, 1970) and the NMR Technique (Morariu and Benga (1977), Conlon and Outhred (1978) and Morariu et al (1985)) and suggests obvious changes in membrane properties that attenuates water flow.

In human cells with a high component of saturated fatty acids in their lipid phase, temperatures above 20-30°C (discontinuity) reduced the water flow considerably implying a membrane state slowing down water movement (Harrison and Lunt, 1980). This is reflected in the high activation energies recorded. As the temperature is lowered, a phase change to a crystalline or more ordered state would be anticipated which, according to the E_a, values favours water movement. This is confirmed by the observation of the % haemolysis for human cells recorded at low temperatures which for the same osmotic stress is far

in excess for that at higher temperatures.

Table IX: Percentage haemolysis at 0.4% NaCl of eel and human, washed and unwashed erythrocytes at 15°C, 25°C and 37°C.

	HUMAN		EEL	
°C	unwashed	washed	washed	unwashed
15°C	90 ±1.16	93 ±0.67	55 ±1.1	83 ±1.1
25°C	89 ±1.0	90 ±0.82	47 ±1.0	65 ±1.0
37°C	31.23 ±1.4	57.13 ±1.5	51 ±1.11	81 ±1.13

Eel membranes, on the other hand, have a much higher unsaturated fatty acid component, typical of ectotherms, and recorded similar E_a (hence water movement character) at their physiological temperature range as endothermic human cells do in theirs. At temperatures far in excess of their physiological range an obvious breakdown of their membrane probably occurs as reflected by the low E_a recorded.

The observations in this study showing that the activation energies for osmotic flow across membranes are low compared to those for bulk water viscosity suggest an assisted water flow across the erythrocyte membrane could be explained by a low degree of interaction between water molecules moving through the membrane under osmotic stress and the membrane itself and is obviously compatible with some form of water slip, described by Vieira et al (1970) at the membrane

water interface. Because low E_a values for water diffusion (NMR method) were not recorded in this work, it is conceivable that the hydraulic pressure brought about by the imposition of an osmotic gradient in osmotic experiments could explain the low values.

It is convenient at this stage to consider the high values of 48-50 KJ mol^{-1} of E_a for water transport across artificial lipid bilayers (House, 1974) which reflects a high degree of water membrane interaction. These high values are required for the water molecule to pass through an energy barrier (or resistance pathway) and is obviously lower in erythrocyte membranes than the artificial lipid bilayers but may well be changed in membranes treated with channel blockers.

2.4.3 Action of Mercurial Compounds on the Activation Energy (E_a)

The temperature dependence of water permeation across human red cell membranes treated with the specific sulphydryl reagent, pCMBS, has been studied by several investigators. Both the osmotic (Macey et al (1972) and Naccache and Sha'afi (1974)) and diffusional studies (Conlon and Outhred (1978), Brahm (1982) and Pitterich and Lawarczeck (1985)) show an increase in the activation energy after treatment. At saturating doses of pCMBS (2 mM) the hydraulic coefficient (Lp) of water crossing human cell membrane is reduced by a factor of 10 (Farmer and Macey, 1970) bringing

it down to values characteristic of lipid bilayer matrix (Haydon, 1969) and suggesting that the mercurial compounds acts by blocking aqueous channels. This is reflected in the activation energy data in which the 13 KJ mol^{-1} of control cells is increased to 31 KJ mol^{-1} in the osmotic study of Naccache and Sha'afi (1974) and from 28 KJ mol^{-1} to 37-40 KJ mol^{-1} in the diffusional experiment of Conlon and Outhred (1978).

In this study, pHMB, demonstrated an opposing effect on the temperature dependence of osmotic haemolysis rates for human compared to eel red cells (Table VIII). The human data in common with the literature confirm the increased E_a of pHMB-treated cells over the normal control values. In the case of eel cells, however, E_a was decreased below the normal eel control values.

For human cells, the increased activation energies are more evident in washed than unwashed cells probably due to the binding of some of pHMB to the extracellular -SH groups (albumin) present in the plasma and hence preventing its full interaction with membrane proteins.

A further observation from the current work on human red cells is that the effects of pHMB are more pronounced at low temperatures and is in agreement with the finding for the diffusional studies of Brahm (1982) who showed that the water transport in human red cells and their ghosts when inhibited by pCMBS leaves a residual or ground permeability

which is similar to that for artificial lipid membranes (Cass and Finkelstein, 1967 and House 1974).

The effects of pHMB appear more obvious at pH 6.8 and 6.4 than at pH 7.4 and is consistent with the data of Naccache and Sha'afi (1974) who suggested the presence of at least two populations of sulphydryl groups which differ in their topical location but both participate in the control of water transport across the cell membranes.

The activation energies quoted in table VIII using pHMB are lower than those of Naccache and Sha'afi (1974) who used pCMBS. This may be attributed to the fact that pHMB is less hydrophilic than pCMBS (due to the presence of the sulphonic acid group) and leads to penetration of some of pHMB through the lipid phase of the cell membrane and hence makes it less effective as an inhibitor.

The reverse effect for pCMBS on eel compared to human erythrocytes was also observed for the NMR water exchange time (table VII). Treatment of human erythrocytes with pCMBS resulted in an increase in the water exchange time (T_2) due to a decrease in the diffusional rate. In eel cells the water diffusional rate was increased in the presence of pCMBS.

Solomon et al (1983) have provided convincing evidence to show that water crosses the erythrocyte membrane through pathways (or pores) which involve proteins (Band 3), a

fact first reported by Macey and Farmer (1970) and Macey et al (1972). All mercurial compounds bind to membrane sulphydryl groups (-SH) with high selectivity.

The findings from the human data can be interpreted in terms of two parallel routes of water transfer - a high energy barrier in a high resistance pathway via the lipid phase appearing under mercurial treatment and a low energy barrier in a low resistance pathway involving proteins containing SH-groups and appearing under normal conditions. In the untreated erythrocytes of both eel and human, the activation energies are low (13-20 KJ mol^{-1}) in agreement with the presence of a low energy barrier (low resistance pathway) whereas, interestingly, in chicken erythrocytes the activation energy for water permeation is 47.7 KJ mol^{-1} (Wieth and Brahm, 1977) indicating the absence of a protein pathway for water transfer, although the integral membrane proteins are quantitatively indistinguishable from the integral proteins of human red cells (Brahm 1982).

The fact that pHMB lowered the activation energy for water movement through eel erythrocyte membranes may be interpreted as an interaction between the mercurial agent and the protein forming pathway by crosslinking the membrane -SH groups leading to their opening and thus a loss of their function as water controlling sites. This implies a different organizational geometry for the protein in these nucleated cells.

As discussed in the introduction (page 15); from Poiseuille's law of viscosity in which

$$P_{os} = N \pi r^4 / 8 \eta_{\omega} \Delta X$$

The osmotic permeability (P_{os}) is related to the geometrical parameter in the membrane ($r^4/\Delta X$) and the viscosity (η_{ω}). Because the literature values for E_a in human cells (Whittembury et al, 1984) and eel (present data) under control condition indicate values for η_{ω} close to those in free solution and since the mercurial compounds do not change the viscosity of water, any change in E_a must be due to geometrical membrane change. This must, in both cases, be due to some conformational change in the protein bound-aqueous pathways leading to their virtual closure or opening through a highly specific interaction of pHMB with the sulphydryl groups. It is therefore suggested that the -SH groups are critical in controlling water movement according to their topical location in the red cell membrane, and that they are different in human and eel. Surprisingly, pCMBS has no reported effect on the water permeation of chicken erythrocyte (Wieth and Brahm, 1977) in spite of the similarity of their integral proteins.

Further characterization and localization of the -SH grouping is necessary and is the subject of the next chapter.

CHAPTER 3

Membrane sites for controlling water passage and their interaction with Sulphydryl compounds

3.1 INTRODUCTION

3.1.1 Effect of Non Sulphydryl Reactive Reagent on Water Transport

A great deal of interest has focussed on the nature of membrane proteins, their interactions with lipids and their functional importance in the regulation of transport across biological membranes. The role of these integral proteins for water transport can, in part, be revealed by treating the erythrocyte membranes with some of the protein fixatives used for histological studies. Formaldehyde, glutaraldehyde and osmium tetraoxide all produce cross-links in proteins and interact with several reactive groups (Molloning and Marinozzi, 1968 and Korn et al, 1972) inducing conformational changes in all proteins of the red cell membrane (Lenard and Singer, 1968). The fixatives, however, apparently do not react exclusively with the proteins, because glutaraldehyde will cross-link phosphatidyl-ethanolamine and phosphatidyl serine, probably to proteins (Wood, 1973), whereas osmium tetraoxide reacts with the double bonds of unsaturated membrane lipids. Despite obvious extensive chemical and conformational changes in membrane protein structure, fixation by formaldehyde fails to change osmotic water permeability (Sirs, 1969) and formaldehyde, glutaraldehyde and osmium tetraoxide (OsO_4) (Brahm, 1982) has no effect on the diffusional water permeability.

Chemicals that reversibly cross-link proteins but not classed as fixatives because they do not denature proteins may be

extremely useful for transport studies. For example, 5,5' dithiobis-(2-nitrobenzoic acid) (DTNB) which inhibits osmotic water permeability by 60% (Naccache and Sha'afi, 1974) and is electrophoretically associated with Band 3 protein (Brown et al, 1975) leads to the suggestion that the water channel is associated with this protein and is obviously not influenced by common fixatives. Brown et al (1975) also showed that the specific anion inhibitor 4,4' diisothiocyano-2, 2' stilbene disulphonate (DIDS) will also bind to band 3 and suggested that water and anion transport probably share a common structural polypeptide chain with perhaps one or more chains specific for the particular functional requirement. In contrast Brahm (1982) showed that DIDS and phloretin (a non specific anion inhibitor) both inhibit anion transport to a level greater than 99% but did not affect water permeability. It was also concluded from this that the anion transport pathway of the integral membrane proteins is unlikely to offer a non-specific leak pathway to water. The lack of effect of phloretin on water transport was explained as being due to an inhibition of water transfer through the proteins and a continuous increase of water movement via the lipid phase caused by a "liquifying" effect on the lipid. Jennings and Solomon (1976) reported on the binding of phloretin to membrane proteins and lipids and found that there was a high affinity binding to proteins and low affinity to lipids. Macey and Farmer (1970) found no effect of phloretin on the hydraulic permeability of water whereas Owen and Solomon (1972) and Owen et al (1974) reported a 30% increase of osmotic water permeability at phloretin concentrations

greater than 0.1 mM. The contradictory evidence from drug binding studies of the proposed aqueous pathway through a protein molecule draws attention to the difficulties encountered when using such "specific" inhibitors.

Although membrane lipids became more fluid when treated with monohydric aliphatic alcohols (Seeman, 1972) such as butanol, hexanol and octanol, the treatment fails to alter their diffusional permeability (Brahm, 1982). This is in direct contrast to a study by Kutchai et al (1980) in which a 40% decrease was observed in osmotic permeability when the hexanol concentration was increased to 18 mM. This inhibitory effect was in fact attributed to an effect of the alcohol on the protein mediated water transport (Brahm, 1982), not the lipid.

Treatment of membranes by some nonelectrolytes (thiourea, glycerol, 1,3-propanediol and dimethylsulphoxide (DMSO) also reduces the diffusional water permeability (Brahm, 1982). The fact that the inhibition was not due to the osmotic properties of the solutes was clearly seen by the fact that the same degree of inhibition was produced by 0.1 M thiourea and 6 M solutions of glycerol, 1.3 propanediol and DMSO. The inhibitory effect of thiourea, glycerol and 1.3 propanediol has been attribtued to their ability to form hydrogen bonds with membrane components in competition with water (Brahm, 1982). However the degree of inhibition by each nonelectrolyte does not appear to be related to their relative hydrogen bonding ability, for thiourea, whose

bounding ability can be expressed by an assigned N value of 4 (Stein, 1967) and having a similar value to that of water, was a considerably more efficient inhibitor than glycerol with an N value of 6. This indicates a larger hydrogen bonding ability. The different efficiences of inhibition may be due, for example, to the inhibitor concentration in the membrane phase.

Small and Goldstein (1982) using NMR showed that equal concentrations of glyceral and DMSO decrease the rate of water transport to virtually the same degree in spite of the fact that DMSO hydrogen bonding ability is lower than that of glycerol (Brahm, 1982). This may be due to the strong interaction with the water molecule through S-O dipoles that probably cause the strong decrease in the self diffusion coefficient of water. Brahm (1982) therefore attributed the apparent inhibition by DMSO not to any interaction with the membrane component but to a reduced self-diffusion of water in the intra- and extra-cellular phase. Furthermore DMSO replaces water in the hydration sphere of membrane bound proteins and is capable of forming stronger hydrogen bounds with the proton-donor groups of the protein than the solvent water. Thus DMSO acts to stabilize the protein configuration to a greater extent than that produced by water.

3.1.2 Effect of Sulphydryl Reactive Reagents on Water Transport

The importance of sulphydryl groups in the maintenance of the structure, integrity and function of the cell membrane of erythrocytes was first suggested by Benesch and Benesch (1954) on producing haemolysis in sheep red cells exposed to organic mercurial compounds. Teleost erythrocytes were found to be much more susceptible to haemolysis from mercaptide forming metals than sheep cells.

Since that time a number of investigations have appeared further demonstrating the importance of membrane sulphydryl groups in water transport control and the fact that it can be inhibited by certain sulphydryl reactive reagents, (Sha'afi, 1981).

Naccache and Sha'afi (1974) in a survey of the effects of sulphydryl reactive reagents (NEM, IAM, pCMBS and DTNB) on water movement in human red cell membrane showed that 1 mM NEM or IAM has no significant effect on water transfer, whereas the same concentration of pCMBS inhibits the rate of water transfer by about 80% and suggested that the membrane -SH groups which are accessible to and reactive with both NEM and IAM were not apparently directly involved in control of water transfer. Naccache and Sha'afi also showed that NEM, IAM and pCMBS react with 60%, 30% and 20% respectively of the total human red cell membrane sulphydryl groups, and suggested that only a small fraction of membrane bound SH groups are directly involved in the control of water transfer. As

mentioned previously, DTNB inhibits osmotic water movement by 60% through disulphide bond formation between membrane SH groups and DTNB. Brahm (1982) showed no effect of DTNB on the diffusional permeability of water and explained this lack of effect by the fact that osmotic transport proceeds via pathways that are not accessible for diffusional transport or at least have a minor role in the diffusion process. It is questionable as to whether this is a likely explanation as both osmotic and diffusional permeability are inhibited by pCMBS in human cells.

There is a similarity for the inhibition of osmotic and diffusional water movement across human erythrocytes using mercurial compounds. Macey and Farmer (1970), Macey et al (1972) and Naccache and Sha'afi (1974) all reported inhibition of about 80% for osmotic permeability in human cells with 1 mM pCMBS at room temperature. Diffusional water permeability data showed an inhibition of about 50% by Macey et al (1972) and Conlen and Outhred (1978), 30-40% by Pitterich and Lawaczeck (1985) who used 1-1.4 mM pCMBS and 60% by Andrasko et al (1976) using 20 mM pCMBS. The results of Brahm (1982) are in general agreement with the previous findings and showed that with increasing mercurial concentration further inhibition appears unobtainable either for osmotic or for diffusional water permeability.

It has been suggested by Brown et al (1975) that the human red cell membrane protein band 3, provides the channel for aqueous transport across the membrane. These authors found

that the sulphydryl reagent, DTNB, binds almost exclusively to band 3 compared to other membrane sulphydryl groups and was the probable site for water transport based on the 60% inhibition obtained by Naacache and Sha'afi (1974). Solomon et al (1983) have also used polyacrylamide gel electrophoresis to show that the predominant site for pCMBS binding to sealed red cell ghosts is on band 3, thus providing further evidence for the proposition that aqueous channels in the red cells are located on this protein (band 3).

As to the binding sites for the drugs on band 3 protein, Toon et al (1985) showed that 1-5 mM DTNB had no effect on osmotic water permeability even when the time of incubation was increased to 3 hours, whereas the use of the sulphydryl mercurials pCMB, pAPMA and FMA, proved that the inhibitory effect previously reported was on both the osmotic and diffusional water permeability. These authors also showed that 5 mM DTNB had no effect on pCMBS induced inhibition of osmotic water transport and concluded that DTNB does not react with pCMBS sites on band 3. The situation however changed when the erythrocytes were pretreated with NEM, for DTNB induced 14% inhibition of osmotic water movement in NEM-treated cells, suggesting that NEM facilitates DTNB inhibition of osmotic water permeability. This is consistent with the work of Benga et al (1983) who reported 8-13% inhibition of water diffusion in NEM-treated cells. In confirmation with previous observations, Toon et al (1985) showed that after NEM treatment DTNB still does not interact with

pCMBS sites.

A new approach in the study of transport processes through erythrocyte membranes is their exposure to proteolytic enzymes. Benga et al (1983) investigated the changes in water diffusion by NMR across human erythrocyte membranes following treatment with various inhibitors and the proteolytic enzymes, trypsin, which digests glycophorin (probably the outer hydrophilic fragment) and chymotrypsin which digests band 3. The results showed that water diffusion was not inhibited by any enzymatic treatment and that the inhibitory effect of mercurials was in no way hampered by proteolytic digestion. In fact the inhibitory effect by mercurials appeared to be slightly potentiated by enzymatic treatment. These findings led Benga et al (1983) to suggest that if band 3 and its associated glycophorin participate in the formation of a water channel, then it must be contained in that part of the protein embedded in the lipid bilayer and not accessible to proteolytic digestion.

Both the organic mercurial reagents pCMBS and the anion exchange inhibitor (DIDS) bind to band 3 and recently Pitterich and Lawaczeck (1985) showed that both reagents have opposite effects on water and proton transport across human and bovine erythrocytes. Their experiments revealed that at concentrations where pCMBS reduces the water permeation by 60-70% the proton permeation is accelerated and that DIDS, which blocks proton-permeation (through H^+/Cl^- cotransport of CO_2 - independent pH equilibration) seems to increase the

water transport rate. DIDS not only lowered the actual proton exchange rates but also lowered the activation energy from 63.6 to 27.6 KJ mol^{-1}. If the passive proton translocation reflects the anion transport then these results are difficult to interpret if both processes use the same principle transport system (Pitterich and Lawaczeck, 1985). Furthermore they suggest that the action of one inhibitor specifically closes one channel and opens a leak pathway at the same time but not necessarily in the same protein. This induced leak is probably connected with other proteins, especially as pCMBS does not exclusively react with band 3 (Solomon et al ,1983).

The inhibitory action of 2.5 mM pCMBS on the osmotic water permeation in the basolateral membrane of the proximal straight tubule of rabbit kidney was also noted recently by Whittembury et al (1984). Its action could be prevented by 0.1 M thiourea. Similarly, Chasan et al (1984) showed that when 0.1 M thiourea was added, with pCMBS, to the erythrocyte suspension it interfered considerably with its action on osmotic water permeability. It was therefore suggested that thiourea acts near the site of pCMBS action. Pratz et al (1986) showed an inhibition of 35% for the osmotic permeability of rat brush boarder tubules by 7.5 mM pCMBS. Most of the osmotic water movement across the kidney membrane is through polar pathways which involve the integrity of the membrane proteins. The only noticeable difference between red blood cells and brush-border membranes was that pCMBS concentration needed for maximal inhibition

differed, 1 mM for the erythrocytes and 7.5 mM for brush border membrane. It was suggested to be due either to the difference in the structure of water and anion channels in the two membranes or to the low number of anion channels in the brush-border membrane.

The effect of the organic mercurials pCMBS can be reversed almost completely by cysteine (10 mM), (Sutherland et al (1967), Naccache and Sha'afi (1974), Conlon and Outhred (1978), Sha'afi (1981), Benga et al (1983), Whittembury et al (1984) and Pratz et al (1986)). The inhibitory action could also be reversed but incompletely or to a different extent by the addition of large protein molecules such as albumin or haemoglobin, Sutherland et al (1967). The reversal of pCMBS action could also be achieved by the addition of glutathione (Weed et al, 1962), and EDTA or penicillamine, (Grimes, 1980).

An important difference in the behaviour of FMA compared to other mercurials became apparent when the reversibility of inhibition by (10 mM - or even 100 mM) cysteine was investigated by Benga et al (1983). FMA was the only mercurial reagent which appeared to inhibit irreversibly the water diffusion through erythrocyte membranes. This feature seems to highlight a difference between the osmotic and diffusional permeability of human erythrocytes, since for the former the inhibition by FMA could be reversed by cysteine (Benga et al , 1982).

The characteristics of the inhibitory sites have been determined by comparing the inhibitory potency of a large number of sulphydryl reagents (Sha'afi, 1977). Based on these studies the following observation can be made:

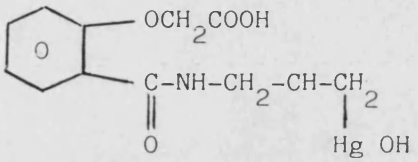
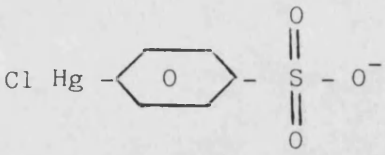
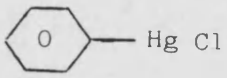
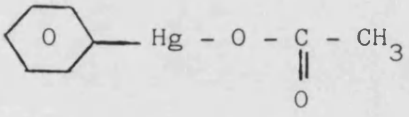
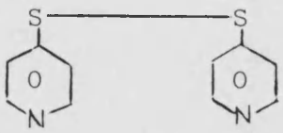
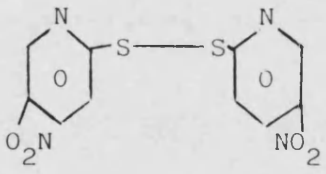
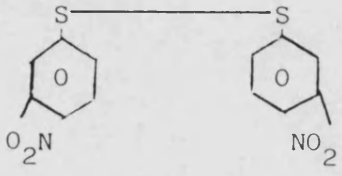
1. Mercury-containing compounds are more effective inhibitors than disulphide reagents.

2. The inability of the compound mersalyl to act as a strong inhibitor of water movement coupled with the finding that mercury-containing compounds are very potent inhibitors of water transport suggest that the -SH groups which are important for water flux are located in a hydrophobic environment. This is supported by the fact that only compounds where the mercury molecule is close to the ring are potent inhibitors.

3. The presence of a nitrogen atom in the ring in addition to an NO_2- group on the side, increases the potency of the disulphide reagent as a water transport inhibitor.

4. Membrane sulphydryl groups which are involved in the control of water transport are less reactive than those of a small -SH-containing molecule such as cysteine.

Effect of various sulphydryl reactive reagents on the water movement across human red cell membrane

Compound	Structure	% of inhibition
Mersalyl		30
p-chloromercuriphenyl sulphonate pCMBS		80
Phenyl mercuric chloride		61
Phenyl mercuri acetate		57
4, 4' - Dithiopyridine		zero
2, 2' Dithio bis-C5-nitropyridine		60
3-Nitrophenyl-disulphide		30

3.2 MATERIALS AND METHODS

3.2.1 Materials

3.2.1.1 Erythrocytes

Erythrocytes from eels and human subjects were collected and washed as previously described in chapter 2.

3.2.1.2 Chemicals

Highest purity grade chemicals were used throughout and were purchased from Sigma Chemical Company. These chemicals were:

a - p.Chloromercuribenzene sulphonic acid, monosodium salt, pCMBS, and used at 1 mM and 2 mM concentrations in eel and human experiments.

b - N-Ethylmaleimide, NEM, used at 2 mM concentrations in both eel and human experiments.

c - Thiolacetic acid (TAA), at concentrations of 1 mM used in experiments with erythrocytes from both eel and human.

d - Cysteine and Bovine Serum Albumin, BSA, used at concentrations of 10 mM and 6×10^{-4}M respectively in human and eel experiments.

3.2.2 Methods

3.2.2.1 Haemolysis Technique

The relative haemolysis rate due to osmotic water permeation across eel and human erythrocytes was determined by the previously described haemolysis technique in chapter 2. The relative haemolysis rate is quoted as the ratio of the experimental value to the control value, this type of representation illustrating the difference between the treated and control cells (Naccache and Sha'afi, 1974). Experimental runs were conducted at least twice on each blood sample and the means used in data analysis.

3.3 RESULTS

3.3.1 Human Erythrocytes

The time course for the pCMBS effect on the relative rate of haemolysis due to the osmotic inward water movement at pH 7.4 was studied at 15°, 25° and 37°C and the results presented in figure I. pCMBS inhibits osmotic water transfer through human red cell membrane at all temperatures.

The degree of inhibition of water flow develops slowly at 15°C, whereas at the higher temperatures the inhibition occurs at a much fater rate. Maximum inhibition occurs between 15°C and 25°C, while at 37°C, although the inhibition of osmotic movement of water develops rapidly, it is less than at the lower temperature and returns to control levels within 90 minutes. At this temperature there is a considerable level of haemolysis observed, whereas the levels are insignificant at other temperatures.

Bovine serum albumin (BSA) partially reverses the effects of pCMBS on the relative osmotic haemolysis by desorption of the drug from the external sulphydryl binding sites. This fact has been used to investigate the role played by the internal sulphydryl permeability sites in water movement by incubating human erythrocyte membranes in 1 mM pCMBS at 25°C pH 7.4 for 40 minutes to attain about 60% inhibition of water movement, followed by the addition of 6

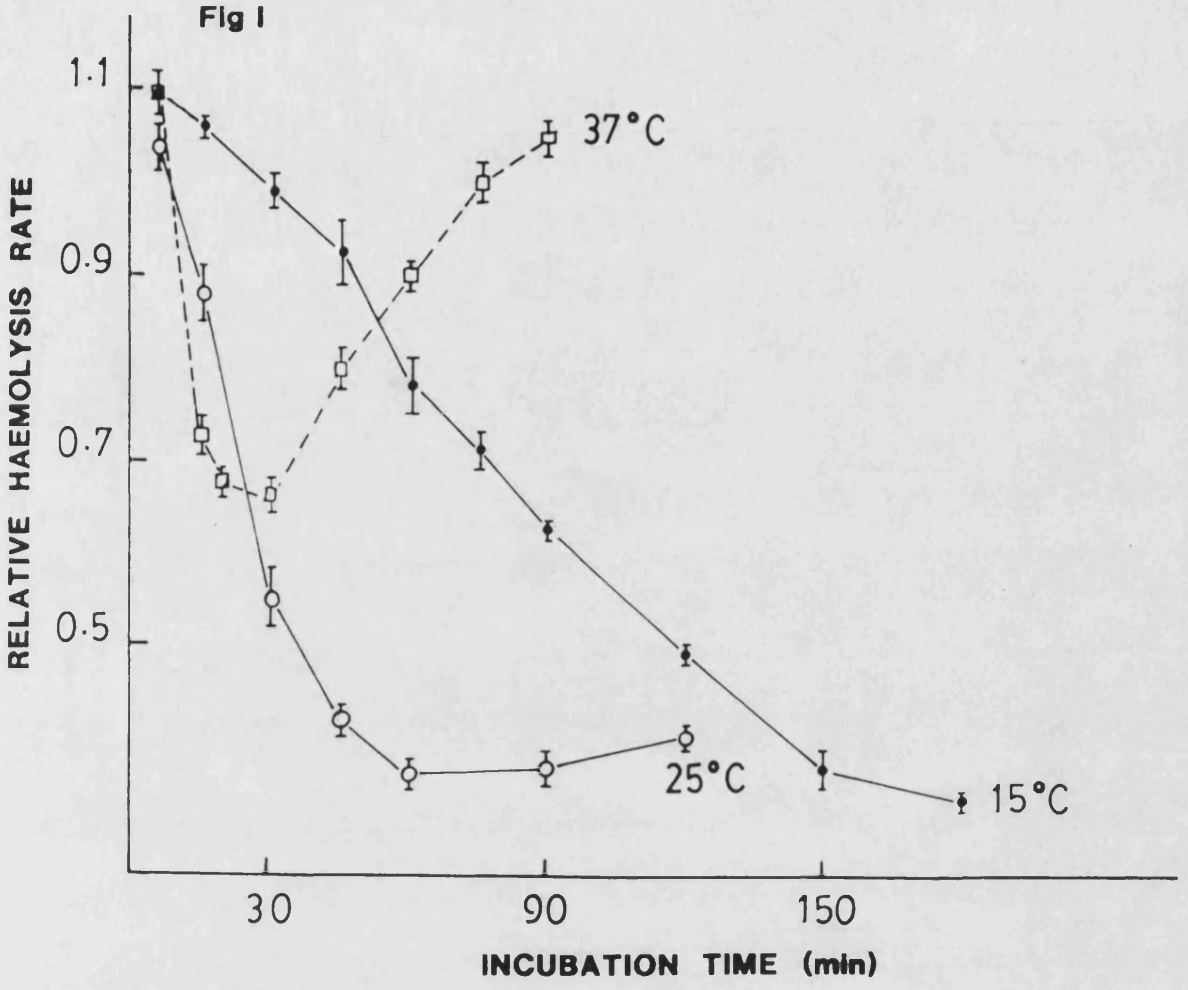


Fig I Relative Haemolysis Rate (RHR) with temperature due to osmotic water flow across Human erythrocyte membranes under the influence of pCMBS at pH 7.4.

$$RHR = \frac{\text{Haemolysis rate after treatment}}{\text{Haemolysis rate for control}}$$

x 10^{-4} M BSA. The results are graphically presented in figure II. With the addition of BSA, the effects of pCMBS on the relative osmotic haemolysis rates can be attributed to the inhibition brought about by the internal SH-groups. It appears from the graph that the relative haemolysis rate was inhibited to a maximum level of about 20% to the total inhibition of pCMBS-treated cells. That is the internal -SH groups are responsible for 20% of water movement across human erythrocyte membrane.

Rao (1979) has demonstrated the presence of groups of sulphydryl sites on the cytoplasmic surface of human erythrocyte membrane which were thought to be protected by pCMBS against NEM binding and vice versa. Steck et al (1978) and Ramjeesingh et al (1980) have suggested, however, the presence of a pCMBS-sensitive, NEM-insensitive site on the membrane and further proposed that this NEM-insensitive site may play a crucial role in the water pathway across the membrane.

In order to investigate both the presence and importance of the NEM insensitive sulphydryl groups in human erythrocyte membrane, 2 mM NEM (N-ethylmaleimide) was incubated with the red cells for one hour at 25°C (pH 7.4) to block all the cytoplasmic -SH groups of the erythrocyte membrane from interacting with pCMBS then 1 mM pCMBS was added to the NEM treated cells. Since NEM was included, the effect of pCMBS on the osmotic water transfer should be attributed to the NEM-insensitive SH groups. The results are

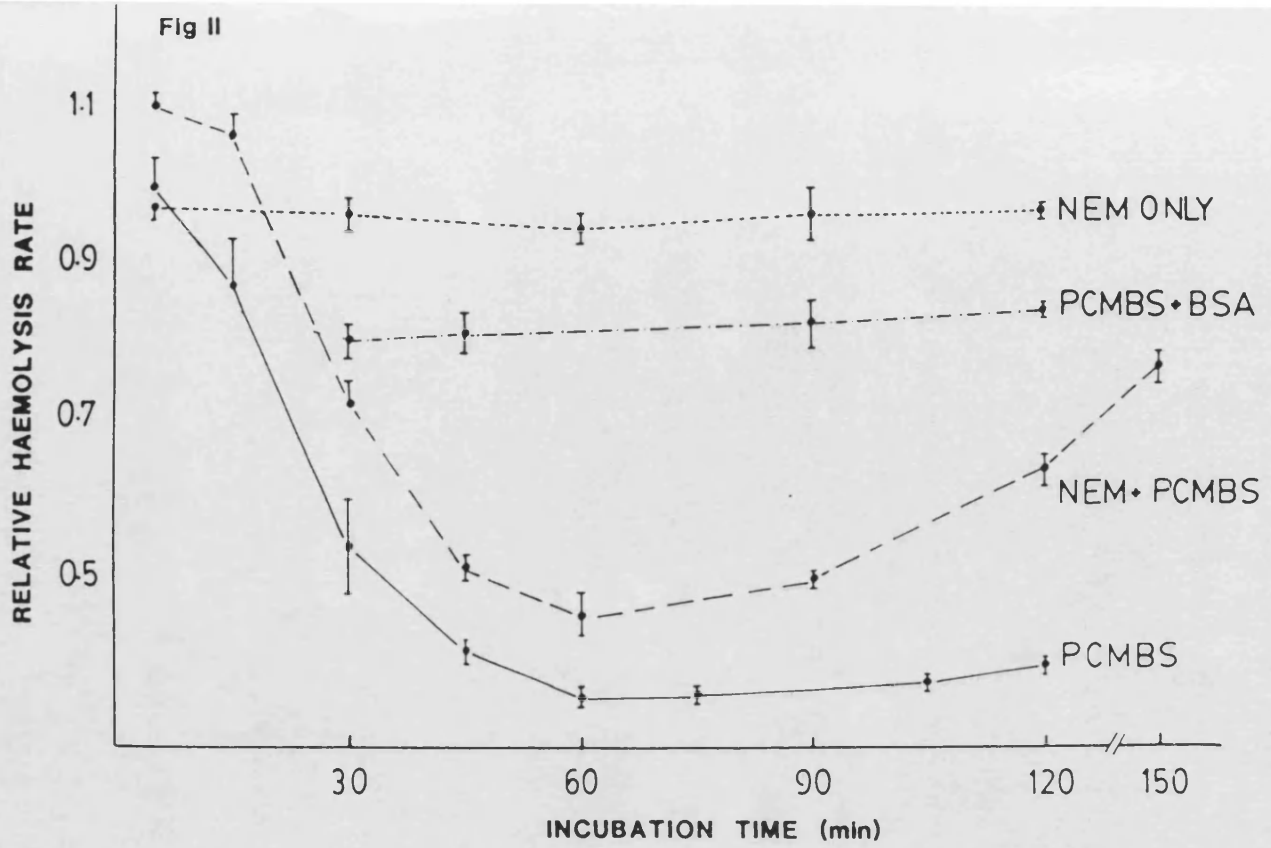


Fig II Effect of pCMBS and NEM on Relative Haemolysis rate in human erythrocytes at 25°C and the reversal effect of BSA on pCMBS action. (BSA added 40 min post pCMBS treatement).

graphically illustrated in figure II. It is clear from the figure that the relative osmotic rate of haemolysis due to water flow was inhibited to a maximum level of about 80% of the total inhibition obtained with pCMBS-treated cells. This suggests that the NEM insensitive sulphydryl groups are the main sites controlling the water transfer through human erythrocyte membrane.

NEM alone had no effect on water flow during the 3 hours of the experiment suggesting that the NEM-sensitive groups (cytoplasmic groups) have no role in the water transport process.

In one experiment shown in figure III, one mM thiolacetic acid (CH_3COSH) was incubated with a human erythrocyte suspension at 25°C for 1 hour, then washed, and 2 mM pCMBS added. The thiolacetic acid is a small molecule which can diffuse and interact with unsaturated compounds (hydrocarbon chain).

It is evident from the data that the osmotic haemolysis rate relative to the untreated control showed initial inhibition which develops to a degree less than that inhibition observed in the case of pCMBS only treated cells. This is followed by an increasing water flux which continues until haemolysis occurs at times in excess of 150 minutes.

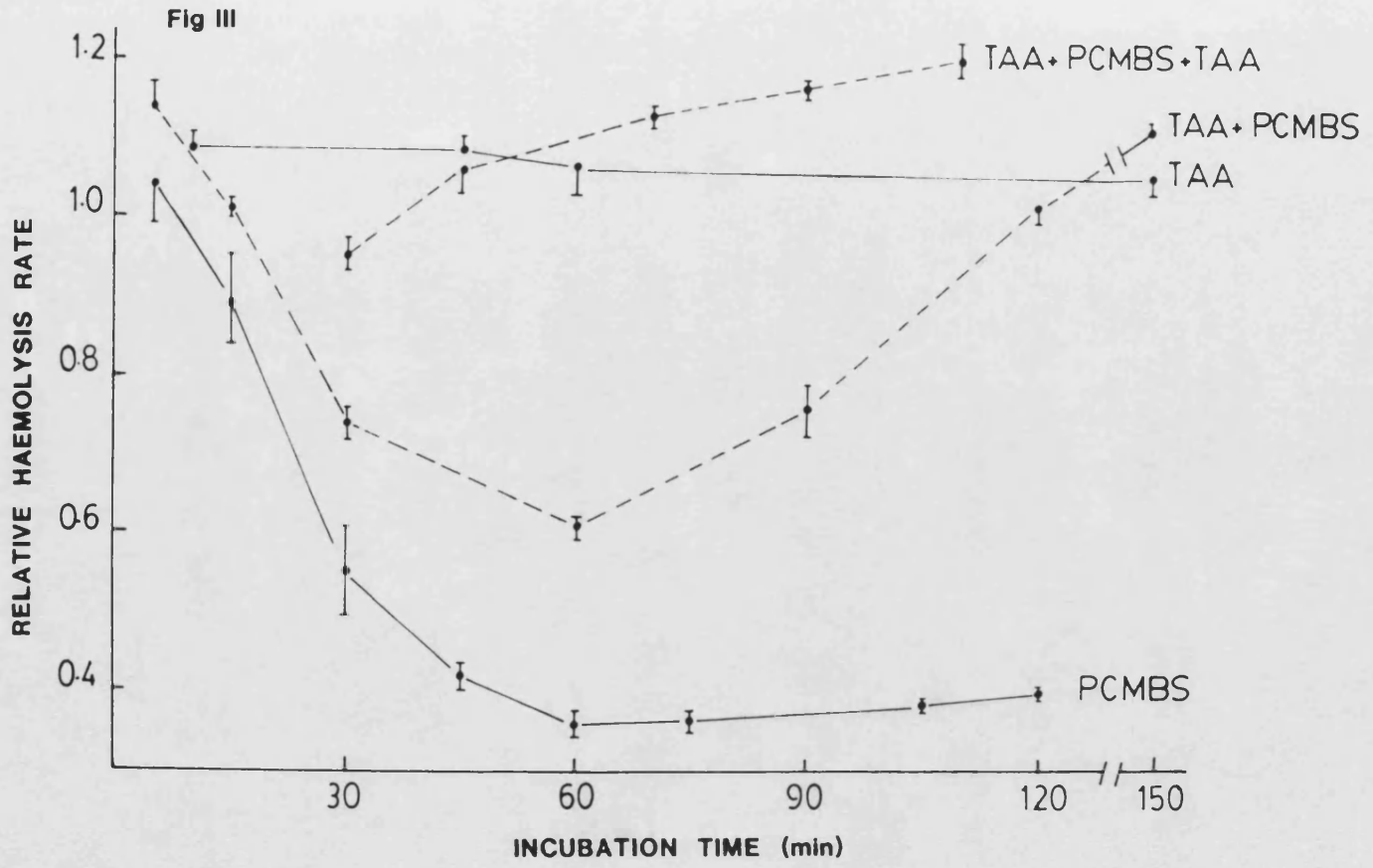


Fig III Relative Haemolysis Rates due to osmotic water transport across human erythrocyte membranes treated with 1 mM thiolacetic acid and followed by pCMBS treatment at 25°C.

The addition of 1 mM TAA during the inhibition phase, produced a rapid recovery of water flow followed by an increase relative to untreated erythrocytes.

The thiolacetic acid alone shown an insignificant change in the relative osmotic haemolysis rate observed during the experimentation period.

3.3.2 Eel Erythrocytes

The kinetics of the rate of osmotic haemolysis due to the inward movement of water across eel erythrocyte membrane incubated with 1 mM pCMBS for 30 minutes was investigated at 15°C and 25°C and pH 7.4.

Figure IV shows the results of a typical experiment for washed and unwashed erythrocytes treated with pCMBS at 15°C. The relative change in water permeability, unlike that of human cells, brings about an increasing haemolysis rate in washed cells compared to unwashed cells. pCMBS-treated unwashed erythrocytes recorded little change from the untreated control. This is probably due to the presence of plasma containing sulphydryl groups competing for the pCMBS and reducing its interaction with those on the membrane.

To investigate the role of external sulphydryl groups on permeability, Bovine serum albumin, BSA, (6×10^{-4}M) and cysteine (10 mM) were added, 40 minutes post-pCMBS

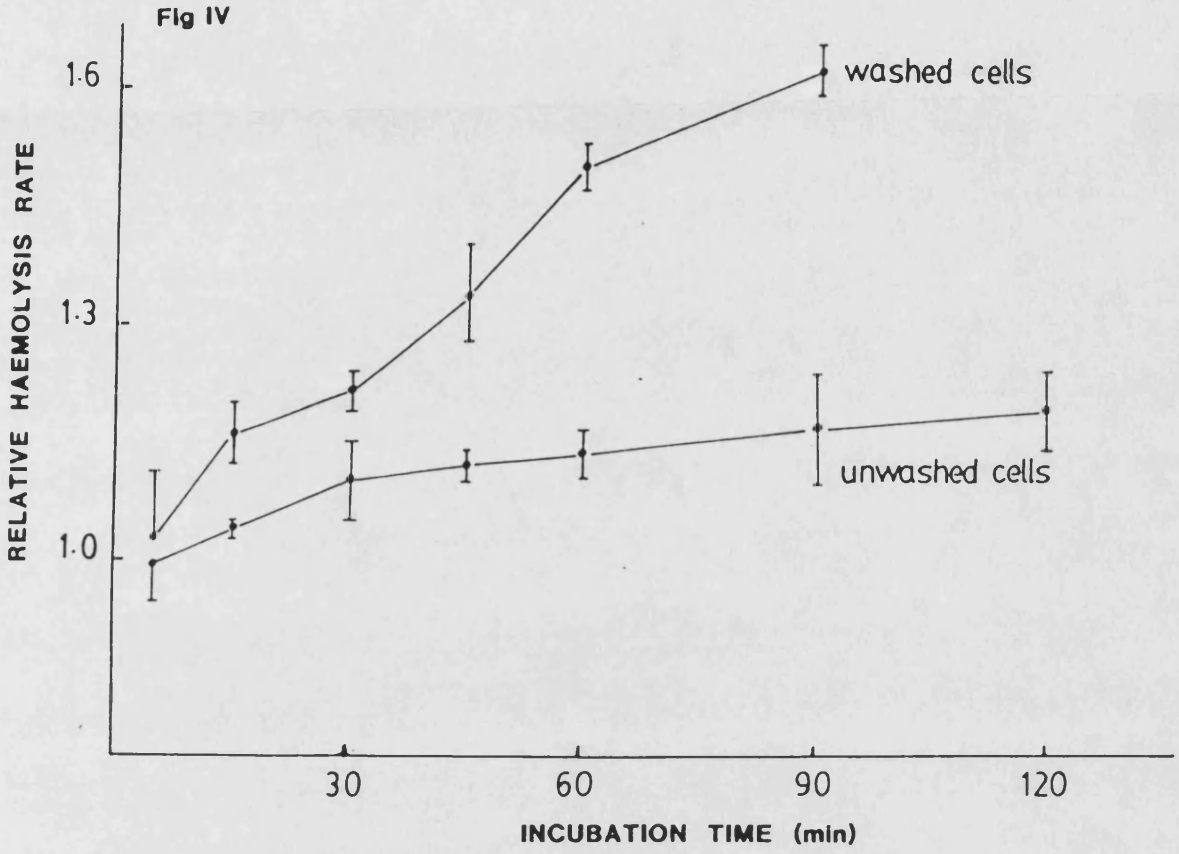


Fig IV Relative Haemolysis Rate due to osmotic water movement across washed and unwashed pCMBS treated eel erythrocyte membranes at pH 7.4 and 15°C.

treatment, to the red cell suspension at 15°C and 25°C. The results are illustrated in figures V and VI respectively. At both temperatures, BSA reduces by approximately 50% the increased relative haemolysis rate brought about by pCMBS treatment alone. The addition of 10 mM cysteine showed complete and rapid reversal of the measured rate at both 15°C and 25°C and a return to the normal control level. This reversal is perhaps more obvious at 25°C than 15°C.

The difference in the level of the recovery after cysteine addition compared to that for Bovine serum albumin (BSA) may be attributed to the location of the permeability sites on the membrane. It appears accessible to the small penetrating molecules like cysteine but not to the large proteins like BSA. This obviously implies that the permeability sites are distributed both inside and outside the membrane. Because BSA is a nonpenetrating molecule it will desorb pCMBS from the external sites only, leaving the internal sites still bound to pCMBS. The 50% increase observed in the relative haemolysis rates must therefore be due to the internal permeability sites (-SH groups) that are still loaded with the drug. Cysteine being a penetrating molecule will desorb pCMBS from both external and internal sites, returning water permeability to control levels.

The N-ethylmaleimide-insensitive permeability sites were investigated by pre-incubation of eel red cells with 2 mM NEM for one hour at 15°C and 25°C (pH 7.4), followed by

Relative Haemolysis Rate due to osmotic water movement across pCMBS-treated washed eel erythrocyte membranes and the reversal by 0.01M cysteine and 6 x 10^{-4}M BSA.

Fig V **at 15°C**

Fig VI **at 25°C**

Fig V

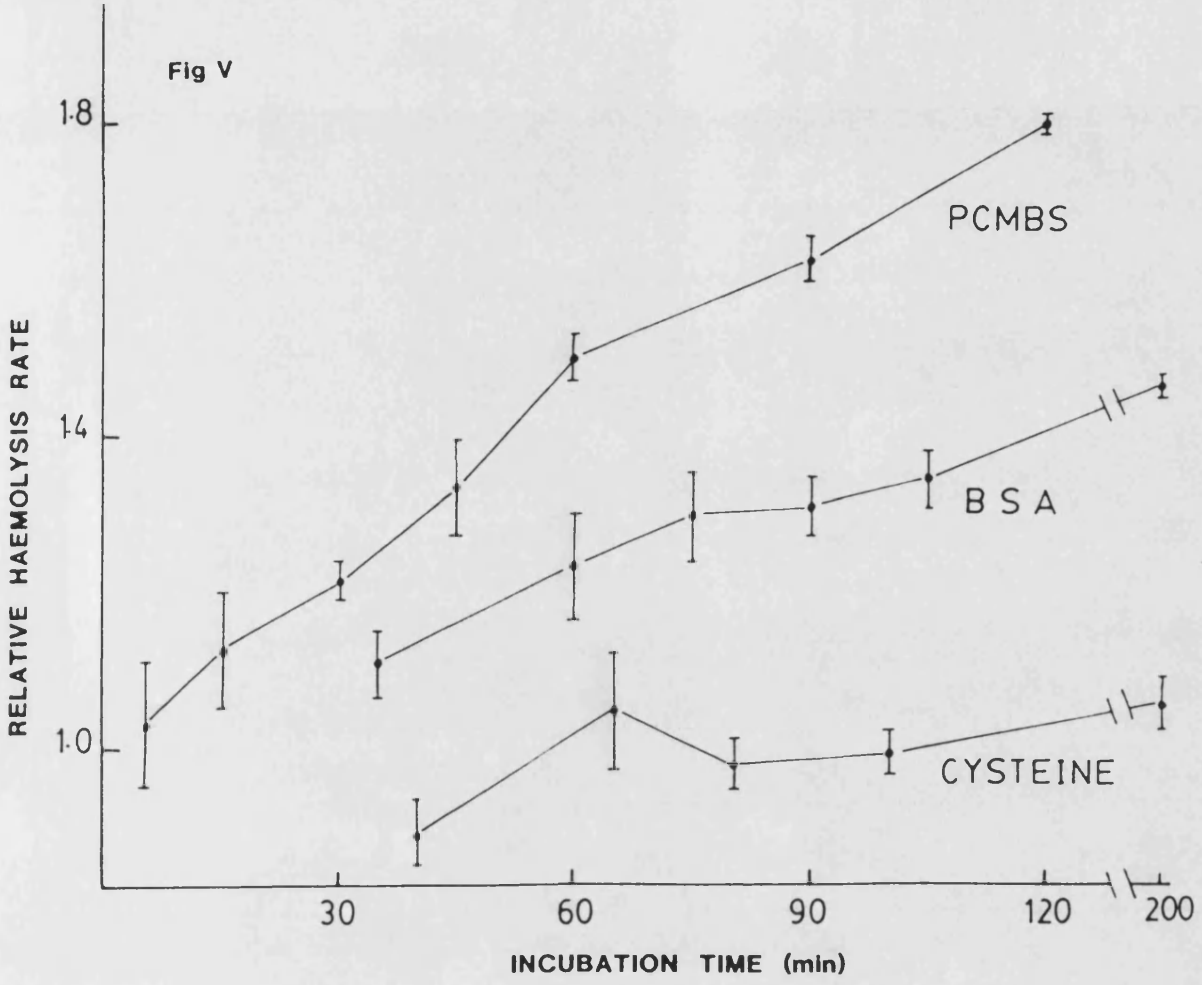
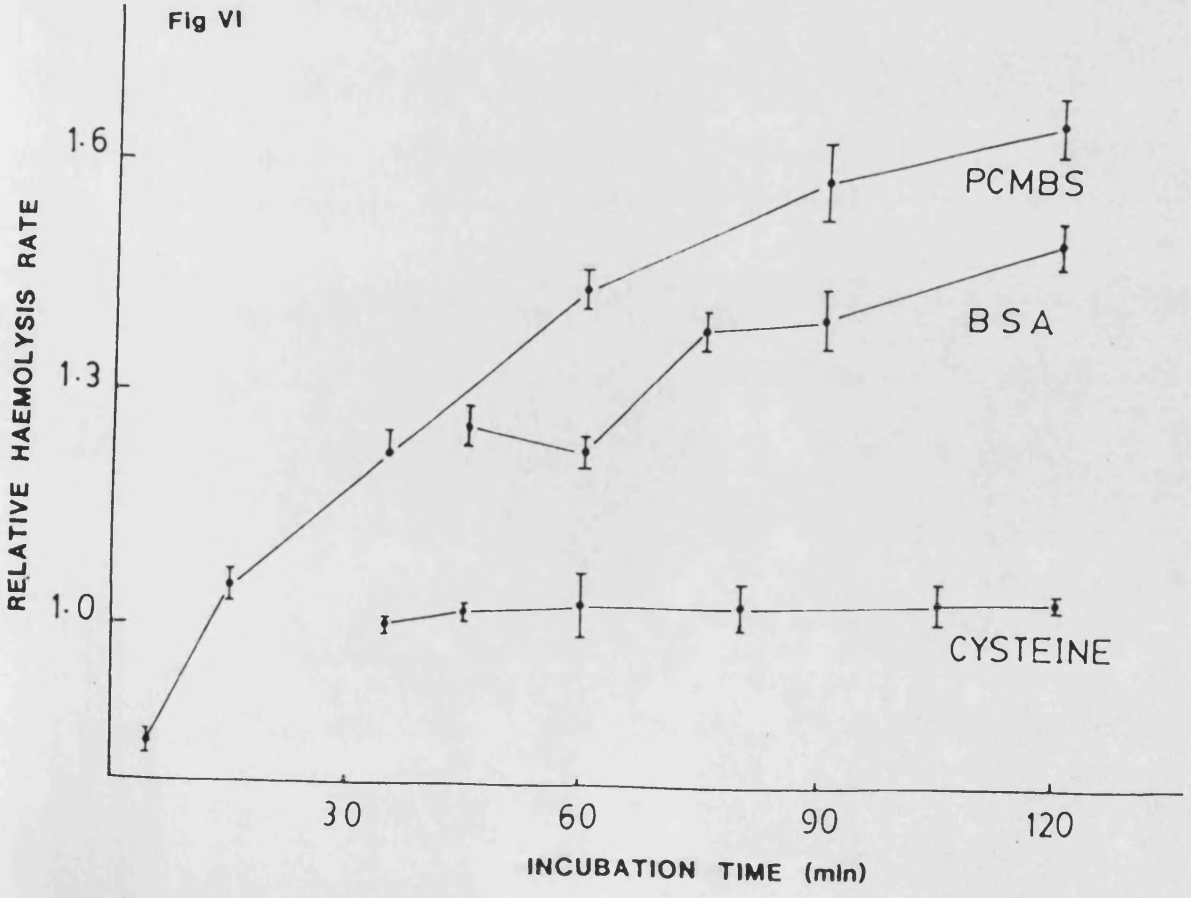

1·8
1·4
1·0
RELATIVE HAEMOLYSIS RATE
PCMBS
BSA
CYSTEINE
30
60
90
120
200
INCUBATION TIME (min)

Fig VI


1·6
1·3
1·0
RELATIVE HAEMOLYSIS RATE
PCMBS
BSA
CYSTEINE
30
60
90
120
INCUBATION TIME (min)

the addition of 1 mM pCMBS. In human cells, when NEM reacts with the membrane sulphydryl groups it blocks subsequent reaction with pCMBS (Rao et al, 1979). The results for eel cells are illustrated in figures VII and VIII and show that the relative haemolysis rates, due to osmotic water transfer, were increased to a maximum level of 95% of that increase recorded in the case of pCMBS-treated eel erythrocytes. This increase could be attributed to the sulphydryl groups which are NEM-insensitive.

On its own NEM showed little reduction in osmotic water flow throughout the experimental period.

Figures VII and VIII also includes the results of the addition of BSA to pCMBS alone-treated eel erythrocyte for comparison.

At this stage, it appears that eel proteins, unlike the human, are considerably different in their water transporting sites.

Fig VII and VIII

Effect of pCMBS on relative haemolysis rate due to osmotic water movement across NEM-treated eel erythrocyte membranes and the reversal by cysteine and BSA.

Fig VII at 15°C

Fig VIII at 25°C

Fig VII

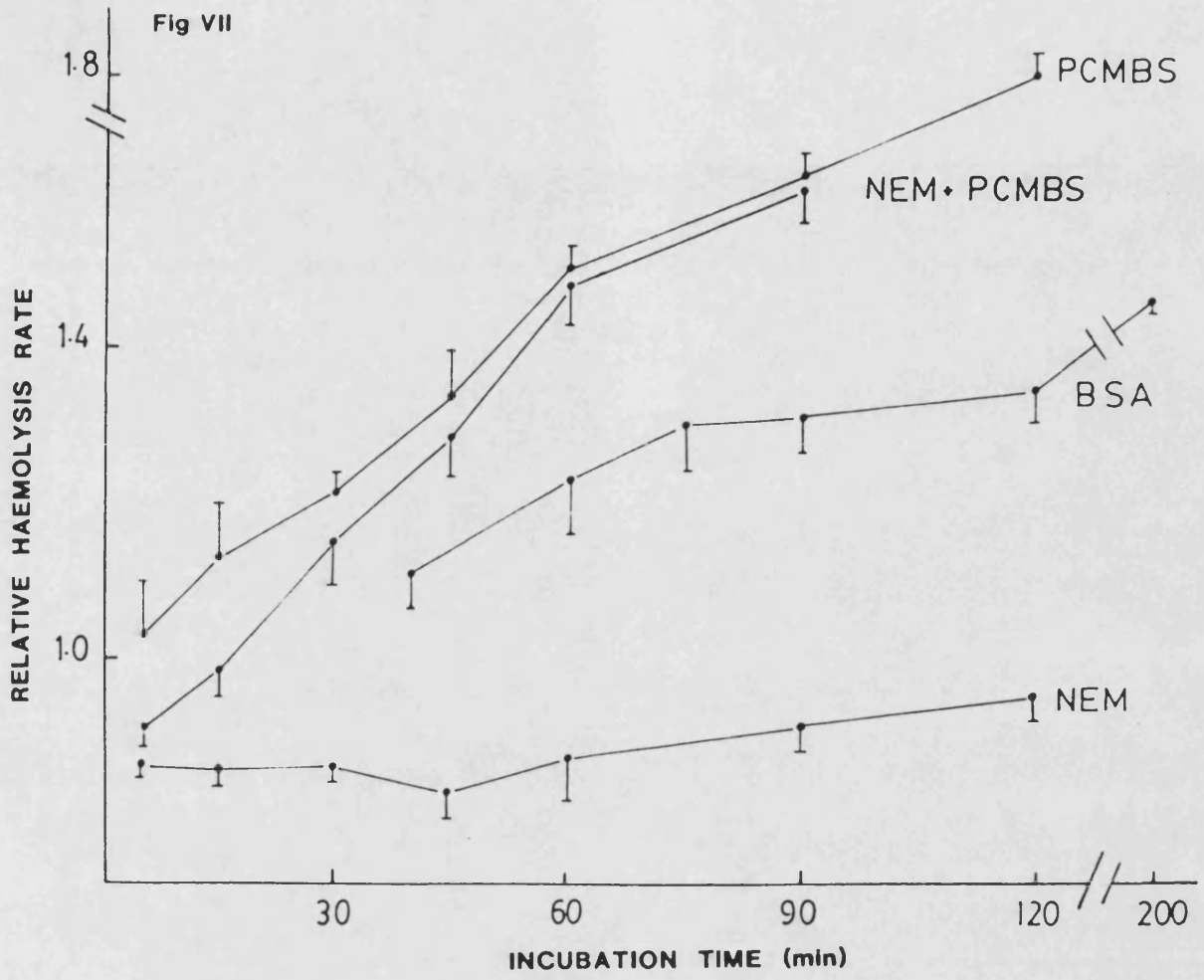
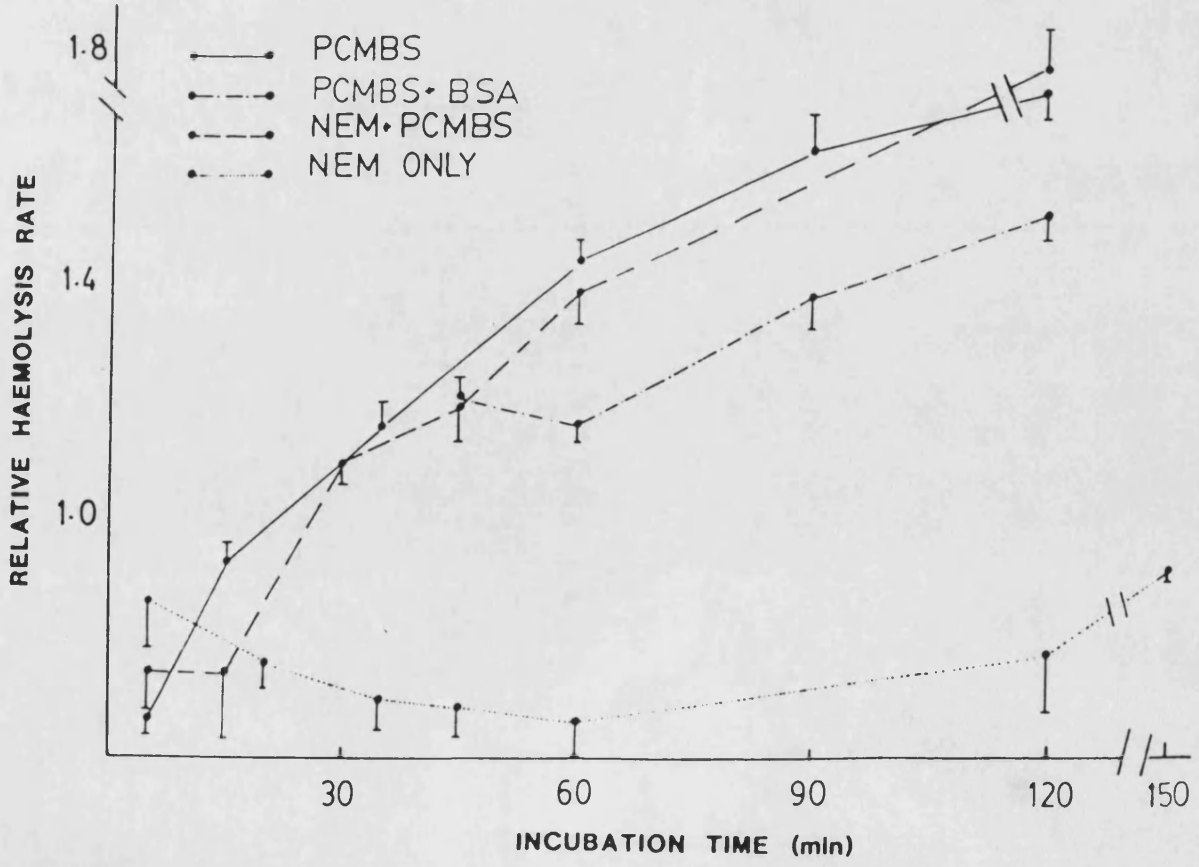


Fig VIII



Figures IX and X show the effect of pre-incubating eel erythrocyte suspensions with 1 mM thiolacetic acid (TAA) at 15°C and 25°C for 1 hour. The thiolacetic acid is a small molecule which can diffuse and interact with unsaturated compounds (in the membrane) and perhaps add extra -SH groups to the molecule. After the incubation period, 1 mM pCMBS was added to the red cell suspension.

The data show a significant increase in the rate of water movement compared to pCMBS on its own (Fig IV).

TAA appears to intensify the action of pCMBS or increase the reactivity of the sulphydryl groups on the erythrocyte membrane. Addition of a further 1 mM TAA at this stage reversed the action of the pCMBS probably due to the desorption of pCMBS. The addition of TAA at 25 °C (Fig X) was five minutes post pCMBS and recovery to control levels was found to be almost complete, while at 15°C, TAA was added one hour post pCMBS treatment and probably due to the late addition, recovery was incomplete (fig IX).

Thiolacetic acid alone showed an insignificant increase in the water movement measured as osmotic haemolysis rate relative to the control (untreated) during the whole experimental period.

Fig IX and X

Effect of pCMBS on relative haemolysis rate due to osmotic water movement across thiolacetic acid treated eel erythrocyte membranes.

Fig IX at 15°C

Fig X at 25°C

Fig IX

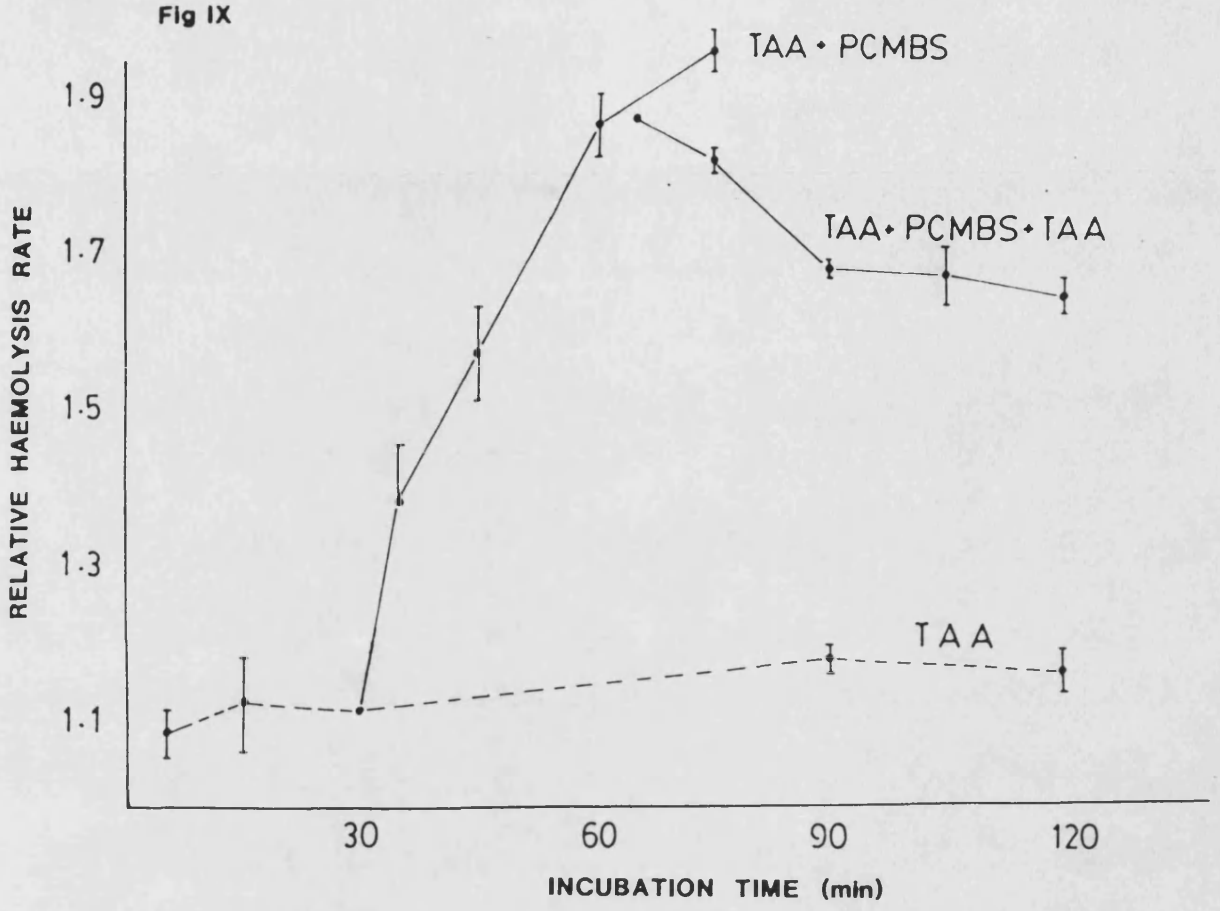
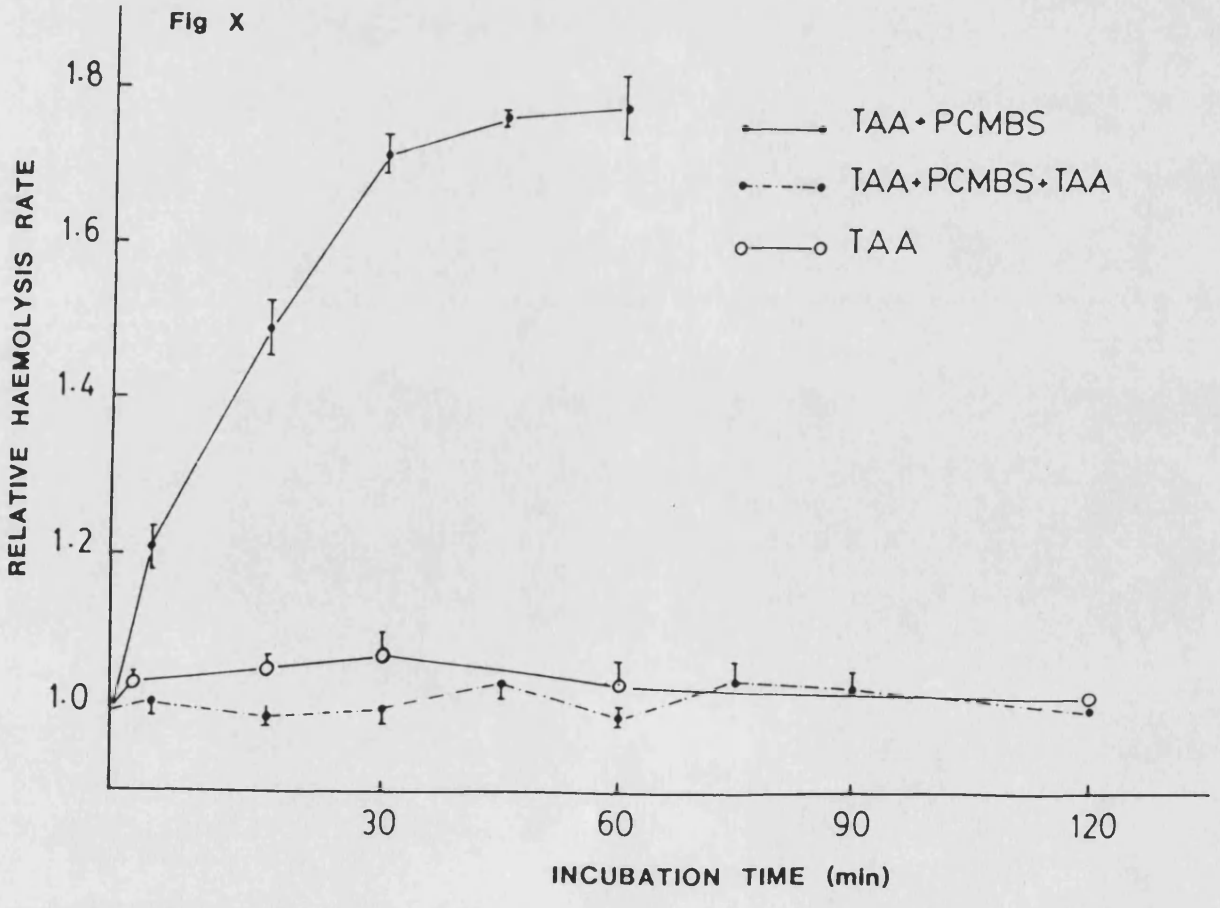

TAA + PCMBS
TAA + PCMBS + TAA
TAA
RELATIVE HAEMOLYSIS RATE
1·9
1·7
1·5
1·3
1·1
30
60
90
120
INCUBATION TIME (min)

Fig X


TAA + PCMBS
TAA + PCMBS + TAA
TAA
RELATIVE HAEMOLYSIS RATE
1·8
1·6
1·4
1·2
1·0
30
60
90
120
INCUBATION TIME (min)

3.4 DISCUSSION

Changes in the permeability of erythrocytes produced by organic mercurial compounds which bind to membrane sulphydryl (-SH) groups have been previously demonstrated by Weed et al (1962), Vansteveninck et al (1965), Shapiro et al (1970) and Morariu et al (1985). Although pCMBS, parachloromercuriphenyl sulphonic acid, is a specific sulphydryl reactive reagent, (Benesh and Benesh, 1962 and Naccache and Sha'afi, 1974), limited attention has been paid to the role of these groups in the control of water transport. Macey and Farmer (1970) and Morariu et al (1985) demonstrated a significant inhibition of both osmotic and diffusional water permeabilities in human erythrocytes with the drug.

The results of this investigation similarly show a significant inhibition of osmotic water permeation through human red blood cells, the full inhibitory effect and the degree of the inhibition being dependent on temperature. At 15°C, 70% inhibition was reached 150 minutes post pCMBS-treatment, 65% inhibition at 25°C after 60 minutes treatment and 35% inhibition 30 minutes post treatment at 37°C. These findings are in agreement with Brahm (1982) who reported that the degree of inhibition produced by pCMBS was more pronounced at the low temperature being 87% at 6°C and gradually reduced to 50% at 38°C. They are also in accord with the thermodynamics of drug binding and are consistent with the fact that pCMBS permeability in human

red cell membranes is low (Naccache and Sha'afi, 1974).

For eel erythrocytes, the reverse was the case in that pCMBS accelerates the water transfer across the cell membrane which continues until haemolysis occurs. This finding supports the earlier observations of Benesh and Benesh (1954) who showed that teleost erythrocytes are much more susceptible to haemolysis when treated with mercaptide forming metals than sheep cells.

The obvious species difference recorded in this work may be due either (i) to the difference in interaction between pCMBS and membrane -SH groups on the eel and human erythrocytes or (ii) a difference in the concentration, reactivity, or location of the SH groups on the two erythrocyte membranes.

The first possibility is unlikely by virtue of the fact that cysteine reverses the effect of pCMBS inhibition or acceleration back to normal in both cases. The reversal is very rapid being less than 5 minutes in both eel and human. Since cysteine itself is a sulphydryl compound that binds easily to pCMBS and desorbs it from the membrane, one can assume that there is little difference between the interaction of pCMBS and -SH groups on eel and human erythrocyte membranes

This investigation shows that the addition of bovine serum albumin (BSA), resulted in 50% and 80% recovery of the

accelerated or inhibited water permeation in eel and human erythrocytes respectively (fig II and V). The difference in the degree of the recovery after the addition of cysteine compared to that with bovine serum albumin probably reflects the location and the function of the permeability sites on the erythrocyte membrane. The internally located sites are accessible to the penetration of small molecules like cysteine but not to the non-penetrating proteins like BSA. It is suggested that there are two classes of permeability sites with respect to their location and function in the membrane that participate in the control of water transfer. Since BSA is a large non-penetrating molecule, it can only desorb pCMBS from the external sites leaving the internal sites loaded with pCMBS, therefore one can conclude that the internal sulphydryl groups are responsible for the 50% acceleration and 20% inhibition of osmotic water movement across eel and human erythrocyte membranes respectively.

A similar finding for human cells was observed by Naccache and Sha'afi (1974) suggesting, from the time course for the development of the inhibition and its dependence on pH, that there are at least two populations of SH groups participating in water movement regulation and differing in their topical location.

To evaluate the role of NEM-insensitive sulphydryl groups in water permeability, some of the membrane sites were blocked with NEM, a compound that can easily diffuse

through the permeability barrier and react with cytoplasmic sulphydryl groups and block subsequent reaction with pCMBS (Rao et al, 1979). This obviously leaves the NEM-insensitive group available for pCMBS binding. The present data demonstrate the 80% inhibition and 90% acceleration of water movement recorded in human and eel red cells, suggesting that the NEM-insensitive SH-groups of both cells appear to be the major sites for the control of water transport and that the NEM-sensitive -SH groups do not participate in water transport. Again this finding is consistent with the data of Solomon et al (1983) for human cells who showed an 80% inhibition in water flux for pCMBS-only treated red cells and 84% in NEM treated cells (NEM + pCMBS). They suggested that if the inhibitory effect of pCMBS on water permeability is due to a reaction with sulphydryl groups, then these groups are NEM-insensitive. This is further confirmed by the fact that NEM on it own had no effect on the water permeability of both human and eel erythrocyte membranes and is consistent with the data of Naccache and Sha'afi (1974). These latter authors explained that NEM, being an uncharged and lipid soluble molecule, might be expected to diffuse through the lipid regions of the membrane and thus not interfere or interact with a protein bound water transfer mechanism, whilst, pCMBS, being an anion and hydrophilic molecule, will select the polar region and interfere in some way with water transport. In this connection, Vansteveninck et al (1965) has shown that substitution of the sulphonic acid group for the Carboxyl of pCMB to yield

pCMBS increases the aqueous solubility of the compound hence increasing the hydrophilic properties and consequently minimising its penetration through the lipid phase of the cell membrane and producing effects related only to the sulphydryl groups of the protein phase.

The internal SH groups in eel erythrocyte membrane demonstrated greater importance (50%) in water transfer than that of human erythrocyte (20%). This could well be attributed to a difference in their relative concentration and distribution at the internal surface. This is explored further in chapter 4 with the use of ^{203}Hg labelled pCMBS.

Robinson (1966) working with brain microsomes as a model for membrane systems has demonstrated that water permeability is increased and ion discrimination decreased by two classes of compounds: 1) those that react with membrane sulphydryl groups, (pCMB) and 2) those that initiate peroxidation (saturation) of membrane lipids (iron plus ascorbic acid). These two classes of compounds are inter-related by the fact (Robinson 1965) that lipid peroxidation increases the content of reactive sulphydryl groups in the membrane, whereas agents binding to sulphydryl groups accelerate the onset of lipid peroxidation (saturation). Although this relationship between reactive sulphydryl groups and reactive double bonds may be secondary to other factors that are more directly related to permeability changes, it is important to consider a possible structural and functional

interaction between sulphydryl groups and lipid double bonds that could underlie the observation in this study and also could be related to physiological and pharmacological membrane events.

Because of this, the thiol compound, thiolacetic ($CH_3.CO.SH$) was allowed to react with eel and human erythrocytes. Thiolacetic acid can interact with unsaturated compound such as olefinic hydrocarbons, series of aliphatic olefins, cyclic olefins and unsaturated carboxylic acid and aldehydes, (Brown et al, 1951). The present data (figs III, IX and X) show that thiolacetic acid had an insignificant effect on water permeation of osmotically stressed human and eel erythrocytes, but the addition of pCMBS to the treated system intensified and accelerated the water movement over pCMBS only treated eel erythrocytes. In contrast, the addition of pCMBS to thiolacetic acid treated human cells showed an initial inhibition (to a lesser degree than pCMBS only treated cells) then an influx of water occurred.

A possible explanation for the considerable increase in water permeability in both cells is that the thiolacetic acid saturates the double bonds in membrane fatty acids increasing conformational changes and destabilizing the membrane which probably leads to an increase in reactive sulphydryl groups available to pCMBS binding. The increase in the number of sulphydryl groups available to pCMBS would obviously lead to a change in membrane structure and hence

Chapter 4

Distribution of Sulphydryl groups in eel and human erythrocytes: ^{203}Hg-pCMBS Binding Experiments

a change in permeability.

This hypothesis is based on the known fact that the presence of cis double bonds in fatty acids results in the hydrocarbon chains being curved, with less effective close-packing, a reduction in interchain force, and a resistance to penetration in monolayers, (Robinson, 1966). The saturation of a cis fatty acid double bond would be followed by a local change in intermolecular forces and surface properties as well as marked conformational changes.

The occurrence of a labile sulphydryl-double bond interaction in the neighbourhood of a membrane pore, where narrow hydrophilic channels penetrate the lipid layers would no doubt provide the possibility of permeability changes. Even though such interactions may contribute little to general membrane stability, the association between specific protein, lipids and -SH groups located at the strategic sites (the membrane pore) could be effective in controlling membrane permeability.

4.1 INTRODUCTION

4.1.1 Erythrocyte Membrane Sulphydryl Groups

Membrane sulphydryl groups have been identified as having pivotal roles in a variety of physiologically important functions, the evidence for which being based on the fact that many functions associated with cellular membranes are changed by reagents that bind to such groups (Grimes, 1980).

Classes of sulphydryl groups have been defined on the basis of their rates of reaction and accessibility to specific sulphydryl reagents. Electron paramagnetic and magnetic resonance studies using covalently bound spin labels have suggested that there are at least two types of sulphydryl groups in ghosts affecting the mobility of the spin label (Sandberg et al, 1969). More detailed attempts to classify these groups for red blood cells are those of Rothstein (1966) and Grimes (1980), who designated three categories, each associated with different functions:

a. Readily reactive, groups reacting with N-ethylmaleimide NEM, organic mercurials pCMB, pCMBS and $HgCl_2$ and comprising some 7% of total membrane -SH groups. Their inhibition has no effect on ATPase activity nor does it affect Na^+ or K^+ permeability.

b. Partially reactive, constituting about 18% of the total membrane groups. They do not react with NEM but do with $HgCl_2$ and organic mercurials giving ATPase

inhibition and increased K^+ permeability.

c. Masked groups constituting the remaining 75%. They do not react with NEM or organic mercurials but do react with $HgCl_2$. The effects of their blocking on the cell are drastic, producing tanning and denaturation as well as increased Na^+ permeability.

A further extension of this basic classification is that of Shapiro et al (1970) who showed that the erythrocyte membrane contains multiple classes (five) of sulphydryl groups. In their experiments, erythrocytes were exposed to 4 mercurials, p-chloromercuribenzoate (pCMB), pCMBS, chlormerodrin (CM), and 1-bromomercuri-2-hydroxypropane (BMHP) for different intervals of time, at different concentrations and in combination with NEM added before and 2-mercaptoethylguanidine (MEG) and reduced glutathione (GSH) added after the mercurials. They showed that many of the membrane sulphydryl groups that bind to pCMB, pCMBS and CM were not accessible to either GSH or MEG. GSH and MEG only remove a small amount of these mercurials from the cell. Shapiro et al (1970) therefore suggested that some of these GSH inaccessible sulphydryl groups may be buried in the membrane or may even be inside the cell. BMHP appeared to react with sulphydryls in an entirely different way from those reacting with pCMB, pCMBS and CM. The binding was immediate and complete and very accessible to GSH which removed all the mercurial (BMHP).

Experiments involving the use of the organic mercurials, pCMB, pCMBS, and CM have also given clues as to the localization of membrane sulphydryl groups; being either at the outer surface or within the membrane. Vansteveninck et al (1965) showed that pCMBS, which penetrates the membrane very slowly, binds rapidly and reversibly to a small fraction of the sulphydryl groups located on its outer face. Sutherland et al (1967), using pCMBS to disturb the cell's K^+ permeability, observed a difference in its rate of recovery after the addition of cysteine compared to that when protein (albumin) was added. The fact that cysteine, having access to both inner and outer SH groups, removed pCMBS from the membrane faster than albumin, which had access only to the external surface, suggests that the sites of K^+ permeability control must be inside the diffusion barrier.

The total number of binding sites for the organic mercurials pCMB and pCMBS were the same, with chlormerodrin (CM) being three times and inorganic mercury four times greater (Vansteveninck et al, 1965). The difference was attributed to the reactivity of the groups on the haemoglobin molecule which contain 90 per cent of the cell's sulphydryl groups (Weed et al, 1962). The only other sulphydryl component of consequence in binding experiments, besides the stroma, is reduced glutathione which reacts with all the test reagents (Weed et al, 1962). These authors showed that the inorganic mercury ($HgCl_2$) reacts with a larger number of sulphydryl groups in

haemoglobin-free stroma than the organic mercurials (pCMB & pCMBS) which themselves react with approximately 22-25% of the total number of cellular sulphydryl groups. They showed also that the rate of reaction of these mercurials (CM, pCMB, pCMBS) with either haemolysed cells, haemoglobin or ghosts is a rapid one. Incubation with the compounds for 60 minutes produces no more binding than that for 5 minutes incubation.

Although the uptake of pCMB and chlormerodrin by intact cells is slow, the binding continues for over 3 hours (Vansteveninck et al, 1965) or 5 hours for pCMBS (Sutherland et al, 1967). In kinetic terms these authors showed, on incubation, a loss of the agent from the cell after the period of maximum binding. This desorption was interpreted as being due to pCMBS leaving the cell as a result of competition by some extracellular pCMBS-binding substances. A candidate for such competition may well be reduced glutathione which has already been reported by Weed et al (1962) to occur even in the absence of the membrane damage produced by the agent.

4.1.2 Band 3 protein

Although the hydrophobic lipid bilayer of the red cell membrane would appear impermeable to hydrophilic solutes, the transport of water and polar anions and cations is accomplished rapidly across it. This exchange is facilitated

by an integral membrane protein with a molecular weight of approximately 95-97 KDa. It can be localized on sodium dodecyl sulphate polyacrylamide gel electrophoretograms where it is found in the third major band from the top. This explains its designation: the band 3 protein. (Passow 1986).

Human erythrocyte membranes contain a very large number of copies of band 3. It is estimated that band 3 constitutes about 25% of the total red cell membrane proteins, (Fairbanks et al (1971) and Jennings (1984)). Binding experiments with the water inhibitors, ^{203}Hg labelled pCMBS (Solomon et al , 1983) and ^{14}C-DTNB (Brown et al, 1975), have shown that the predominant labelled peak is the one on band 3.

By proteolytic digestion in situ, it is possible to split the band 3 protein into a number of well defined fragments Fig (A)(Passow,1986). Externally applied trypsin produces no cleavage, while internal treatment splits off a hydrophilic 42-KDa section which is released from the membrane, while the remaining 55-KDa hydrophobic section stays in the bilayer. When the red cells are treated with external chymotrypsin, two fragments of 35-KDa and 60-KDa are formed that both remain associated with the membrane. When the chymotrypsin-treated membranes are isolated and then exposed to internal trypsin, the 55-KDa fragment is found to be cleaved into two fragments (35-KDa and 17-KDa) that continue to be associated with the membrane while the

42 KDa piece is detached. The 35 KDa is tightly bound to the membrane (Steck et al (1978) and Jennings (1984)) and crosses the membrane at least twice, (Rao (1979), Rao et al (1979), Ramjeesingh et al (1983) and Jennings (1984)). The 17 KDa peptide must cross the membrane an odd number of times, since its N-terminus is intracellular and its C-terminus is the extracellular chymotrypsin cleavage site (Jennings, 1984). Further reduction of the 35 KDa fragment to 9 KDa is achieved when chymotrypsin is applied at higher concentrations and low ionic strength (Passow ,1986). The hydrophilic 42 KDa peptide with an N-terminus, does not cross the bilayer and faces inwards (Passow, 1986).

External papain splits the protein at three locations, two of which reside in close proximity to the chymotrypsin cleavage site that delineates the 17-KDa and the 35-KDa segments, (Jennings et al (1984) and Passow (1986)). Also low concentrations of external pronase produce two fragments of 60-KDa and 35-KDa which are indistinguishable from the fragment observed after treatment with chymotrypsin (Cabantichik and Rothstein, 1974.b).

Amino acid sequences of the 42-KDa and the 55-KDa domain of murine band 3 have been reported by Passow (1986) and it remains to be seen what variation on this basic theme are in other animal species.

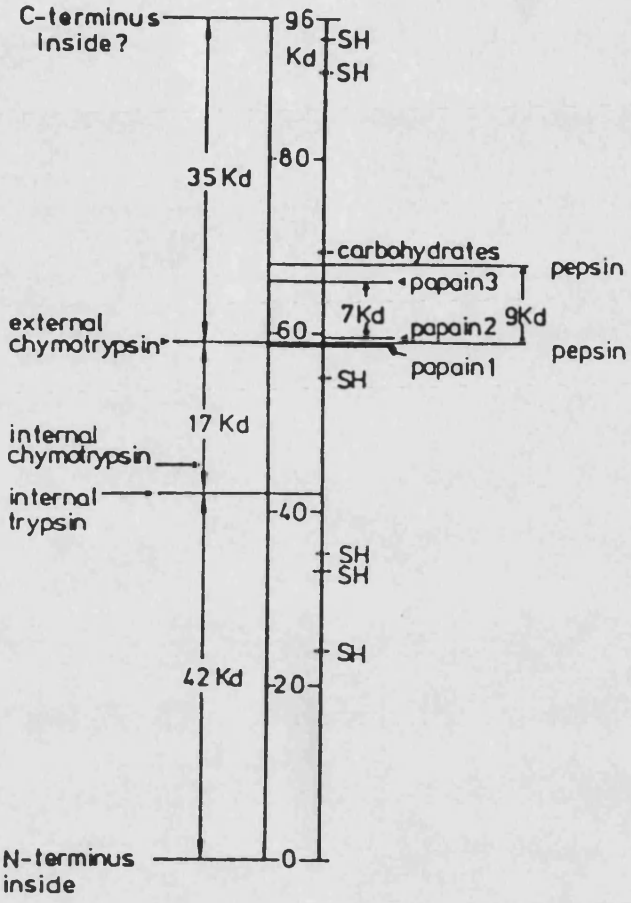


Figure A **Delineation of major proteolytic fragment as obtained after <u>in situ</u> digestion of human band 3 protein. (Quoted from Passow, 1986).** Location of C-terminus is not yet unequivocally known, but possibly located at the inner membrane surface. N-terminus is hydrophilic and does not cross the bilayer and faces inwards.

Band 3 protein extends over a broad molecular weight range on SDS polyacrylamide gel electrophoretogram. This diffuse appearance of the band disappears after treatment with a suitable glycosidase (Jennings, 1984) due to the heterogeneity of carbohydrate moiety of the molecule. Jennings (1984) has reported that each band 3 monomer has a

single carbohydrate chain, which is bound to a single asparagine residue in the C-terminal 35-KDa region of the protein (Drickmar, 1978). It forms single oligosaccharide chains of variable length, the longest containing many repeated galactose-N-acetylylucosamine units, (Tsuji et al, 1980). The carbohydrate has no known role in transport (Jennings, 1984).

Erythrocytes from other species: The peptide patterns of erythrocytes from different species are similar, particularly in the case of ox, sheep, pig, camel, dog, rabbit, guinea pig and rat (Lenard (1970b) and Kobylka et al (1972)) and even the nucleated goose (Shelton, 1973) and chicken erythrocytes (Brahm, 1982). The similarities are apparent in the dominating 95 KDa polypeptide (band 3), which is found in variable amounts in all species with the spectrin fraction (band 1 and 2) (Lenard, 1970b and Kobylka et al, 1972).

The most obvious species differences concerns the glycoproteins, which differ considerably in their carboydrate contents (Hudson et al, 1975). To what extent these differences affect the peptide moiety is not known (Deuticke, 1977).

4.1.3 Distribution of -SH groups on band 3 protein of Human erythrocyte

The presence of sulphydryl groups on band 3 protein was first suggested by the studies of Brown et al (1975) on the binding of the specific sulphydryl reagent DTNB. Confirmation of their presence was also made by Solomon et al (1983) on the binding of pCMBS to band 3.

Rao (1979) and Rao and Reithmeier (1979) have demonstrated five -SH groups on band 3, all being reactive under appropriate conditions, with the sulphydryl reagent, N-ethylmaleimide. Accessibility by the membrane -impermeant maleimide reagent (Abbott et al, 1976) however is only from the cytoplasmic surface of the membrane. The five groups have been classified into two sets of reactive groups, one set having an extracellular site for cleavage with chymotrypsin. Three of the groups are present in a 60 KDa fragment generated from band 3 by cleavage with chymotrypsin at the external site. The other two are present in the complementary 35-KDa fragment. The sulphydryl groups in the 35 KDa fragment are unreactive in ghosts and can be labelled with (^{3}H)-N-ethylmaleimide in cells (Rao, 1979). Evidence from the work of Rao and Reithmeier (1979) show that all three sulphydryl groups with an NH_2 terminal external to the chymotrypsin site are cytoplasmic. They are released from the membrane by trypsin and are located in the 20-KDa fragment that probably represents a sulphydryl contining domain of band

3. The NH_2-terminal of the 23 KDa protein and the membrane bound 19-KDa fragment, produced by chymotrypsin treatment of ghosts, appear to contain no reactive sulphydryl groups (Rao and Reithmeier, 1979). The two reactive -SH groups (located at the -COOH terminal of the external site for chymotrypsin) are contained in a 55-KDa tryptic fragment of band 3 that remains tightly membrane bound. This is clearly shown in Fig (B).

Fig B

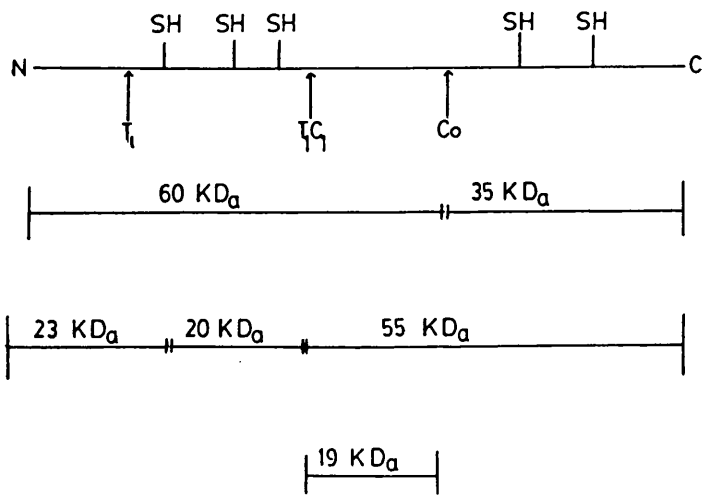


Figure B **A map of human band 3 protein showing the relative positions of the sites for proteolytic cleavage and NEM reactive -SH groups. T_1, cytoplasmic-trypsin-sensitive sites; C_1 and C_0, cytoplasmic and extracellular chymotrypsin -sensitive sites. (Quoted from Rao and Reithmeier, 1979).**

Steck et al (1978) and Ramjeesingh et al (1980) have reported an additional, sixth -SH group in band 3. The studies of the latter authors were devoted to a 15 KDa sequence contained in the 17 KDa membrane fragment that lies between the trypsin and chymotrypsin cleavage sites and contains the binding site for DIDS. Since Rao and Reithmeier showed that there is no NEM reactive groups in the 17 KDa sequence, then it appears that this sixth group does not react with NEM. Solomon et al (1983) denoted it as a cryptic -SH group and considered it to be extracellular to the DIDS binding site (Fig C). The argument to support this location is based on an observation by Solomon et al (1983) that the stilbene anion inhibitor, DIDS, does not alter the pCMBS effect on water transport, whereas it prevents access of pCMBS to the intracellular SH groups. In order to determine whether the sixth SH group is actually located on band 3, Solomon et al (1983) examined the location of the pCMBS site in NEM

Fig C

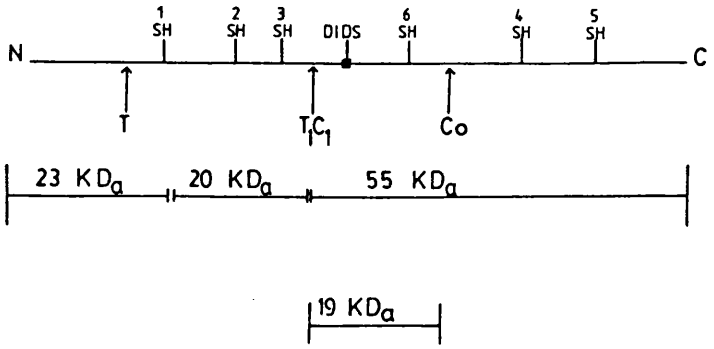


Figure C Schematic diagram showing possible location of the sixth SH group and DIDS binding site

treated human ghosts by polyacrylamide gel electrophoresis using (^{203}Hg) - pCMBS and showed that the predominant peak is the one on band 3. The schematic diagram showing a possible disposition of band 3 and the distribution of the sulphydryl groups in human erythrocyte membrane is shown in Figure D.

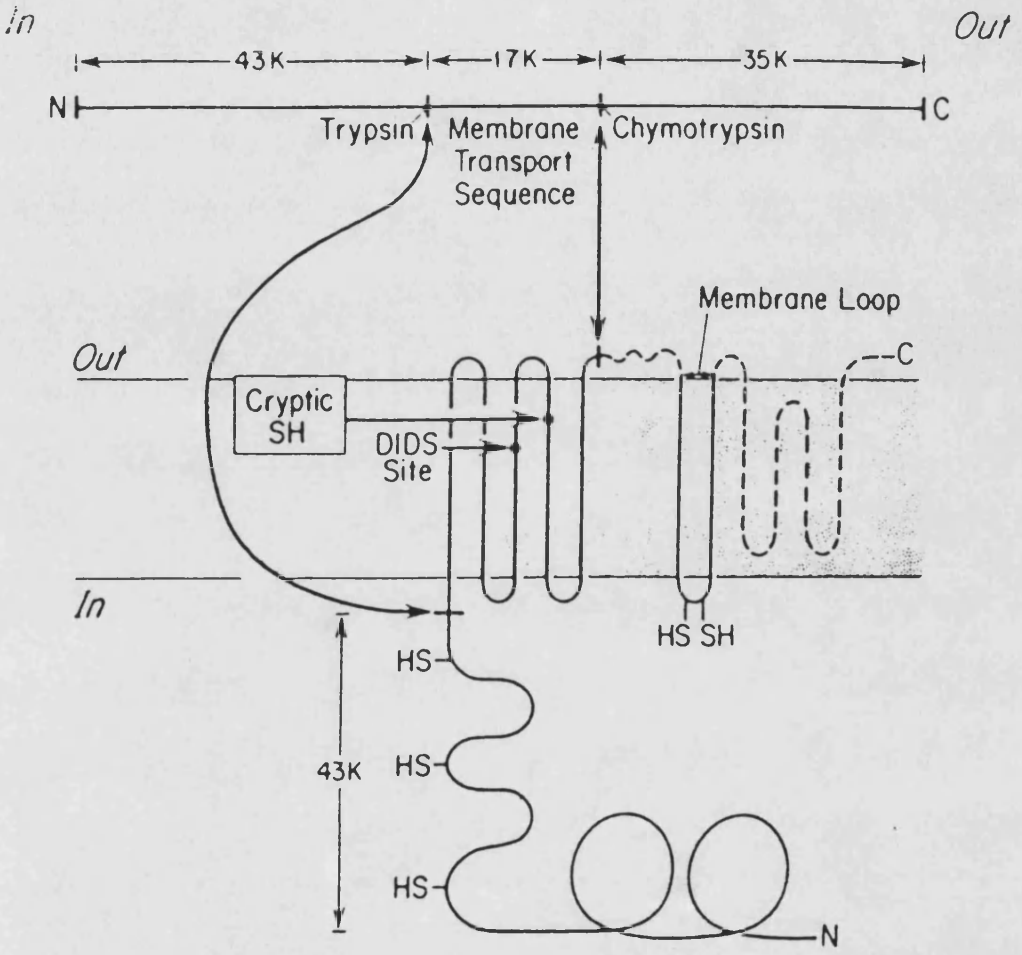


Figure D **Schematic diagram showing possible location of SH groups on band 3 proteins of human erythrocyte membrane. The number of loops is purely arbitrary. (Quoted from Solomon et al, 1983).** It is not known accurately how often the Band 3 traverses the membrane nor whether the C-terminal is extracellular or within the membrane.

Under approriate conditions, Reithmeier and Rao (1979) have shown that band 3 can be cross-linked with Cu^{+2}-o-phenanthroline to form a dimer. The cross-linkining is quantitative and results in the loss of only one reactive -SH group per band 3 monamer. This group must therefore be included in the formation of the intersubunit disulphide bond responsible for cross-linking. This sulphydryl group is located in the 20-KDa fragment (Steck et al (1976) and Rao and Reithmeier (1979)). Analysis of the cytoplasmic domain by Reithmeier and Roa (1979) have shown that all three -SH groups of the cytoplasmic domain contribute equally to the formation of this bond, that is, the cross-linking generated in a population of diamers involve (with equal probability) anyone of the three NEM-reactive-SH groups. The formation of any one intersubunit disulphide bound per dimer must, therefore, preclude the formation of the others. The authors have attributed this effect to a conformational change that occurs upon cross-linking which is consistent with results that show that the kinetics of anion transport (Passow (1986) and water movement, Solomon et al (1983)) are altered upon cross-linking with specific sulphydryl reacting compounds.

4.2 Materials and Methods

4.2.1 Materials

4.2.1.1 Erythrocytes

Erythrocytes from eel and human were obtained and prepared as previously described in chapter 2.

4.2.1.2 Chemicals

As descrbed in chapter 2.

The labelled organic mercurial compound, ^{203}Hg-pCMBS (2.8 GBq g^{-1} Hg), was obtained from Amersham International, England and used at a concentration of 2×10^{-5} M.

4.2.2 Methods

4.2.2.1 Measurements of ^{203}Hg-pCMBS binding to intact erythrocytes

^{203}Hg labelled pCMBS was used to determine the presence and quantity of sulphydryl groups on the red blood cell membrane. The labelled agent was dissolved in isotonic saline solution at pH 7.4 and then added to the cell suspension at 5% Haematocrit to give a final concentration of 2×10^{-5} M (25 K Bq ml^{-1}). It was then allowed to equilibrate for measured time intervals at 15°C or/and 25°C. The supernatant was removed by centrifugation and the cell fraction washed three times with ice cold saline. The amount of ^{203}Hg-pCMBS bound to the cells was calculated

by measuring directly the radioactivity taken up by the cells in a manual well-type Na I crystal counter or an automatic gamma scintillation counter (LKB 1275 minigamma counter). The binding of pCMBS is expressed as mol $cell^{-1}$.

4.2.2.2 Determination of Red Blood Cell Count

Cell counts were performed with the improved NEUBAUER cytometer (Hawksley, London) or using the electrozone celloscope particle counter (model 111 LTS Elmhurst, Illinois, USA).

4.2.2.3 Determination of Haemoglobin Content

Haemoglobin was determined colorimetrically using Drabkin's Reagent obtained from Sigma Chemicals (Poole, Dorset).

4.2.2.4 Determination of Total Binding Sites on Haemoglobin and Erythrocyte membranes

For these series of experiments, fresh blood was collected and washed 3 times in isotonic physiological saline solution, followed by osmotic lysis in distilled water for human or 30 mM NaCl(pH 7.4) for eel erythrocytes. This latter treatment prevents any gel formation which is attributed to either vacuolation or disruption of the nucleus. Centrifugation was then performed at 9000 xg for 15 min at 5°C to separate haemoglobin from cell debris.

The haemolysate was dialyzed for 24 hours at 4°C against 1% NaCl to remove glutathione and any other filterable thiols. The sulphydryl content of this dialyzed protein is virtually equal to that of the haemoglobin, (Vansteveninck et al, 1965). The haemoglobin concentration was determined by Drabkin's reagent.

The cell membranes were washed 3 times in 30mM NaCl solution as described previously. The presence of a nuclear gel occasionally formed during eel cell membrane preparation, was eliminated by dialyzing the stromal phase against 30 mM NaCl for 24 hrs at 4°C, followed by 3 washes in the same concentration of NaCl. Less than 1% of the pCMBS bound to these cell membranes may be attributed to haemoglobin contamination of the stroma (Vansteveninck et al, 1965).

^{203}Hg-pCMBS at concentrations of 6×10^{-5}M were incubated with intact cells, haemoglobin-free stroma and haemoglobin solutions at 15°C.

The amount of binding of pCMBS to intact cells was calculated as described previously.

In the case of haemoglobin-free stroma and haemoglobin solutions, the binding of ^{203}Hg-labelled pCMBS was calculated from the radioactivity in the precipitate after addition of 10% trichloroacetic acid.

4.3 RESULTS

Localization of erythrocyte membrane sulphydryl groups:

4.3.1 Eel erythrocytes

The binding curves for pCMBS and its desorption are shown for 15°C and 25°C in figures I and II.

The curve for 15°C can be divided into 2 component parts (i) an initial very rapid binding which is completed in a few minutes amounting to 26.8×10^{-19} mol pCMBS $cell^{-1}$; (ii) a slower buildup of agents to a maximum binding of 32×10^{-19} mol $cell^{-1}$ at about 3 hours.

The efficiency of extracellular sulphydryl substances on pCMBS desorption was tested directly by adding 6×10^{-4} M Bovine Serum Albumin (BSA) and 10 mM cysteine to the cell suspension 30 minutes post pCMBS addition. These additions resulted in a rapid desorption of pCMBS from the cell membranes. In less than the five minutes necessary for centrifugation and three washing procedures, 10×10^{-19} mol $cell^{-1}$ was desorbed by BSA and 20×10^{-19} mol $cell^{-1}$ by cysteine.

The significant difference in the amount desorbed by the readily permeant cysteine compared to the non-penetrating

protein BSA can be attributed to the location of the sulphydryl sites on the membrane. It appears that 50% of the SH-groups are present on the external surface of the erythrocyte membrane whilst the rest are on the cytoplasmic surface or inside the membrane.

The fraction of pCMBS that did not desorb after the addition of either BSA or cysteine is called the non-desorbable pCMBS and amounts to 14 x 10^{-19} and 3.8 x 10^{-19} mol $cell^{-1}$ respectively. It is assumed that this residual pCMBS has passed through the membrane to combine with the large pool of intacellular haemoglobin.

At 25°C, the binding of the pCMBS to eel red cells at pH 7.4 was found to be very rapid amounting to 21.9x 10^{-19} mol $cell^{-1}$ in less than 5 minutes, followed by a slower buildup to 28 x 10^{-19} mol $cell^{-1}$ over seven hours until haemolysis started.

The addition of BSA 30 minutes post pCMBS incubation, caused a loss of 8 x 10^{-19} mol pCMBS $cell^{-1}$ while the addition of cysteine resulted in 16 x 10^{-19} mol desorbed $cell^{-1}$. This confirms the 50:50 distribution of sulphydryl groups on both sides of the permeability barrier of eel erythrocytes. The non-desorbable pCMBS left after addition of BSA and cysteine was 16 x 10^{-19} mol $cell^{-1}$ and 3.8 x 10^{-19} mol $cell^{-1}$ respectively (ie same as at 15°C).

Fig I and II

Binding of 2 x 10^{-5}M pCMBS to eel erythrocyte membrane sulphydryl groups and desorption by 0.01 M cysteine and 6 x 10^{-4} BSA.

Fig I at 15°C

Fig II at 25°C

Fig I

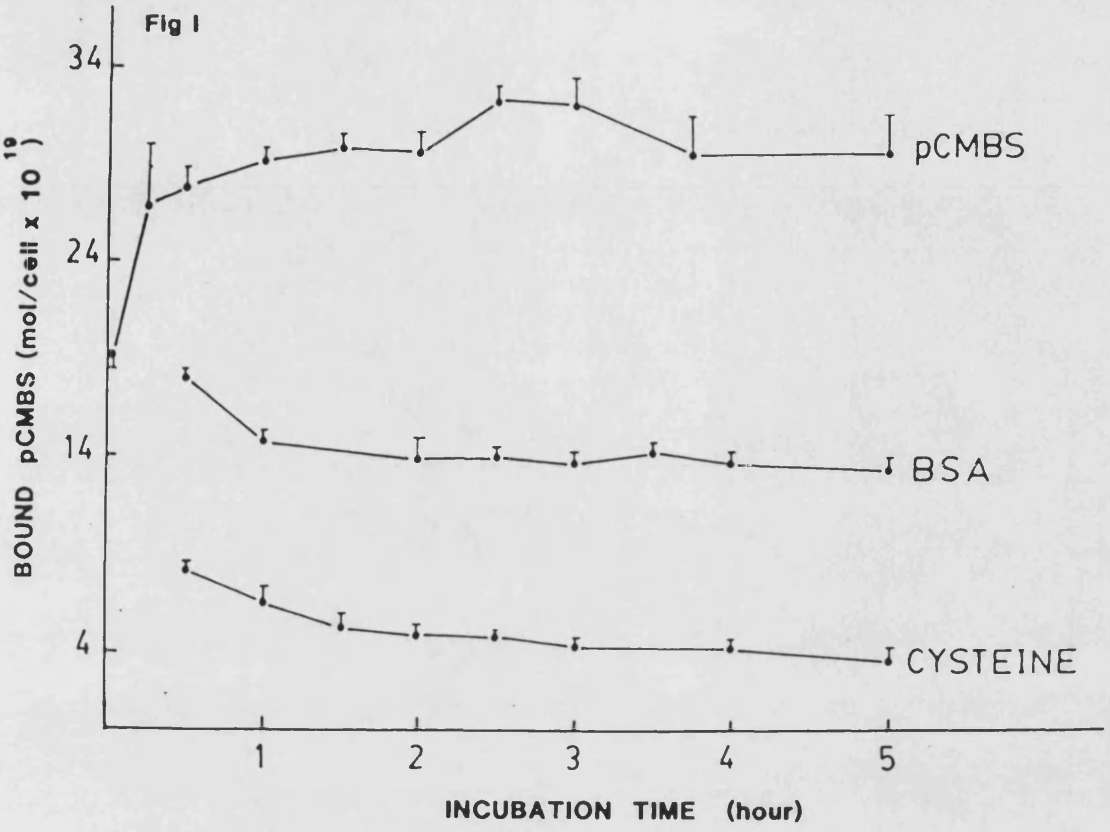
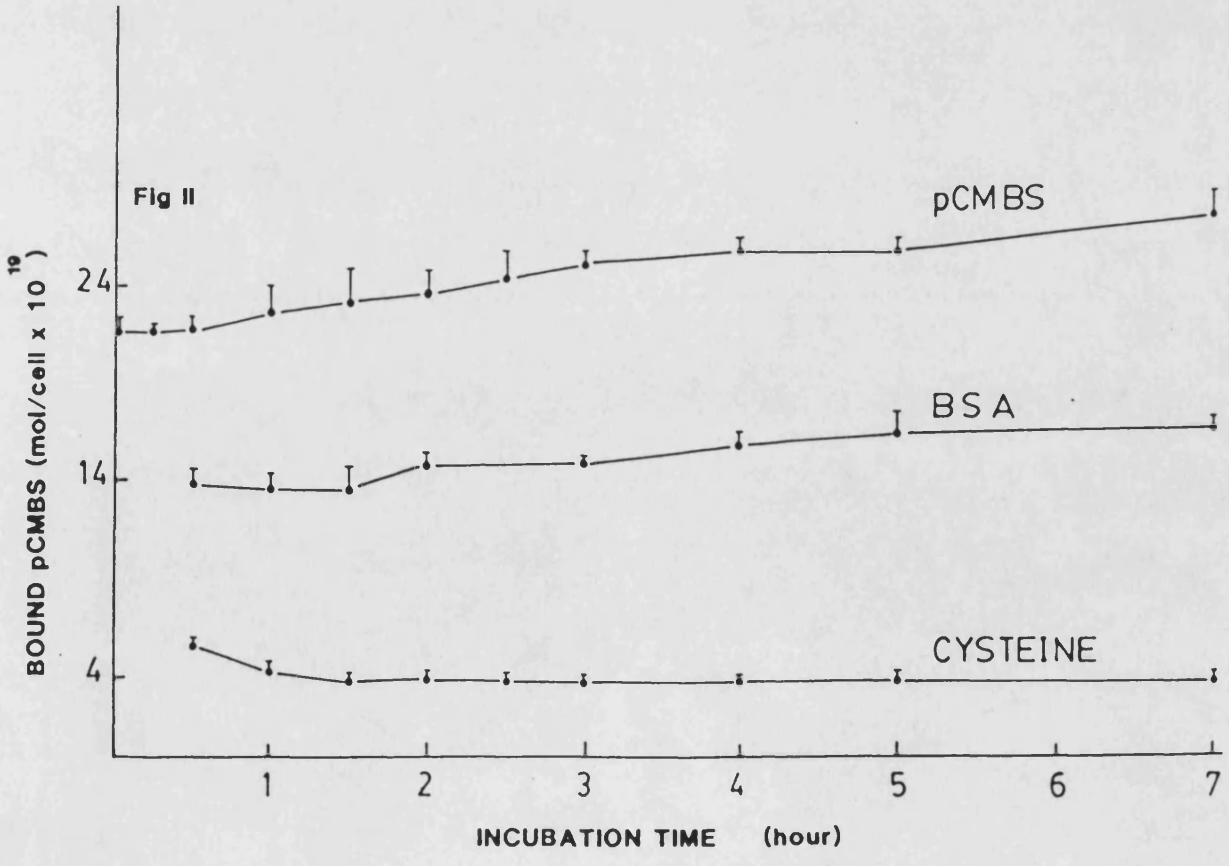

34
24
14
4
BOUND pCMBS (mol/cell x 10 19)
pCMBS
BSA
CYSTEINE
1
2
3
4
5
INCUBATION TIME (hour)

Fig II


24
14
4
BOUND pCMBS (mol/cell x 10 19)
pCMBS
BSA
CYSTEINE
1
2
3
4
5
6
7
INCUBATION TIME (hour)

The effect of N-ethylmaleimide on the binding of pCMBS to the membrane sulphydryl groups is shown in figure III. The NEM was incubated for one hour with eel erythrocytes at 15°C and 25°C pH 7.4, followed by the addition of labelled pCMBS to the cell suspension. Jacob and Jandl (1962) have showed that the uncharged alkylating agent NEM enters the cell freely and becomes bound to the intracellular SH groups of human erythrocyte membrane.

At 15°C the binding curve for pCMBS to eel erythrocyte membrane showed 3 binding steps, (i) an initial slow buildup to a maximum level of 40.8 x 10^{-19} mol $cell^{-1}$ after two hours, which is slightly higher than pCMBS only treated cells, (ii) a maintained level of binding thereafter over not less than five hours and (iii) a significant increase in level of binding to 54 x 10^{-19} mol $cell^{-1}$ reached after 24 hours. A considerable amount of haemolysis was observed after that time.

The addition of BSA to the cell suspension 30 minutes post pCMBS treatment resulted in an initial desorption of 2.8 x 10^{-19} mol $cell^{-1}$ over the untreated control and a slowing down of the binding of the agent for several hours. After 24 hours, however, a very high level of pCMBS binding was recorded reaching that of the NEM + pCMBS treated cell suspension. Haemolysis was also observed at that time.

The addition of cysteine at 30 minutes post pCMBS treatment, resulted in a rapid desorption of 13.6 x 10^{-19}

mol pCMBS $cell^{-1}$ followed by a sustained slow desorption to the end of the experiment at 24 hours with no haemolysis being observed.

The residual non-desorbable pCMBS was about 13 x 10^{-19} mol $cell^{-1}$, higher than in the case of pCMBS only treated cells. It may be presumed that NEM increased the membrane permeability to the pCMBS to combine with the large reservoir of haemoglobin inside the cell.

Figure IV shows the effect of 2 mM NEM on pCMBS binding at 25°C pH 7.4. The binding curve shows three component parts, (i) an initial very rapid binding which is completed in 30 minutes amounting to 31.2 x 10^{-19}mol $cell^{-1}$, (ii) a minimal loss of the agent over 1.5 hours and (iii) a very rapid buildup of the pCMBS over the next 2 hours giving total level of 56.5 x 10^{-19} mol $cell^{-1}$.

The addition of BSA 30 minutes post pCMBS treatment resulted in a small desorption of 3 x 10^{-19} mol $cell^{-1}$ from the cell membrane followed by a sustained level over the next 1.5 hour. There was then a very rapid binding amounting to a level of 56 x 10^{-19} mol $cell^{-1}$ similar to that for NEM + pCMBS-treated cells.

The addition of cysteine immediately desorbed 18 x 10^{-19} mol $cell^{-1}$ and then showed a slow desorption of agent amounting to 13 x 10^{-19} mol $cell^{-1}$ after 4 hours of the experiment.

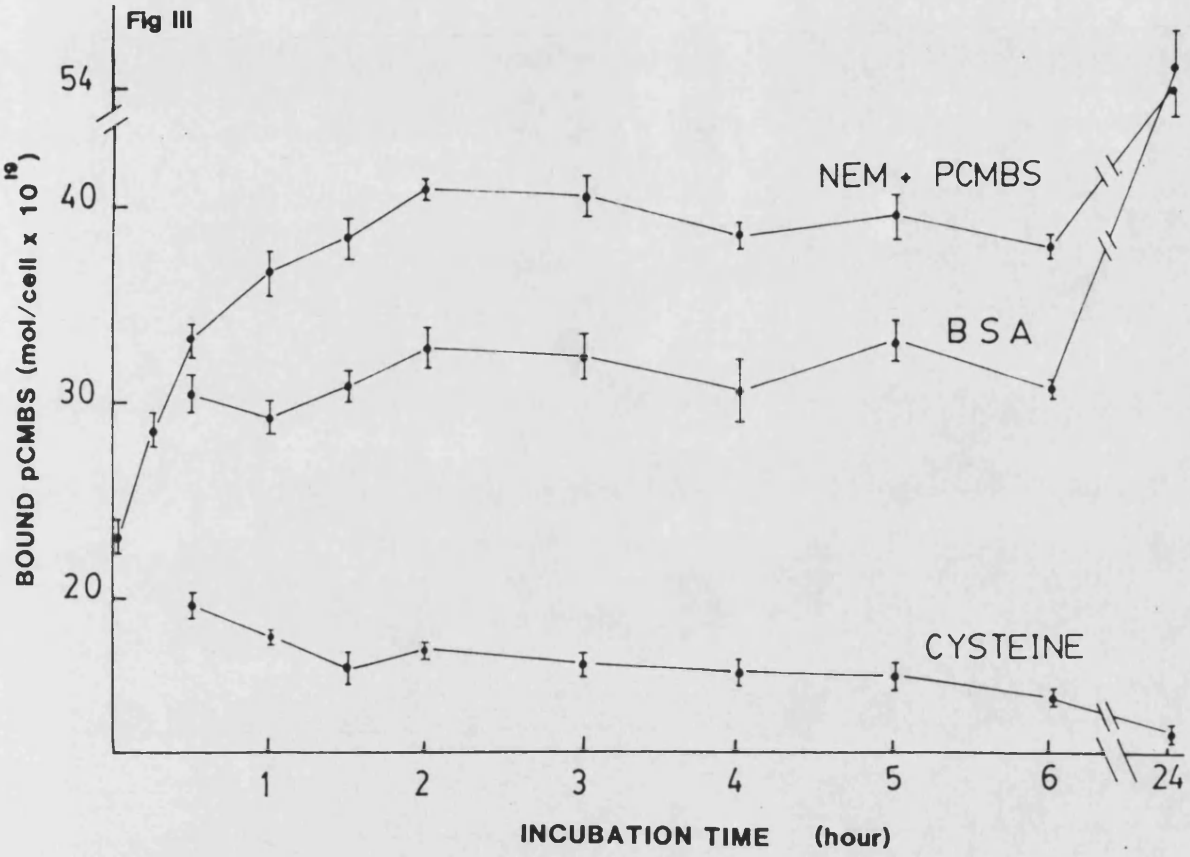


Fig III and IV

Binding of ^{203}Hg-pCMBS to eel erythrocyte membrane sulphydryl groups pretreated with NEM and reversal by cysteine and BSA.

Fig III at 15°C

Fig IV at 25°C

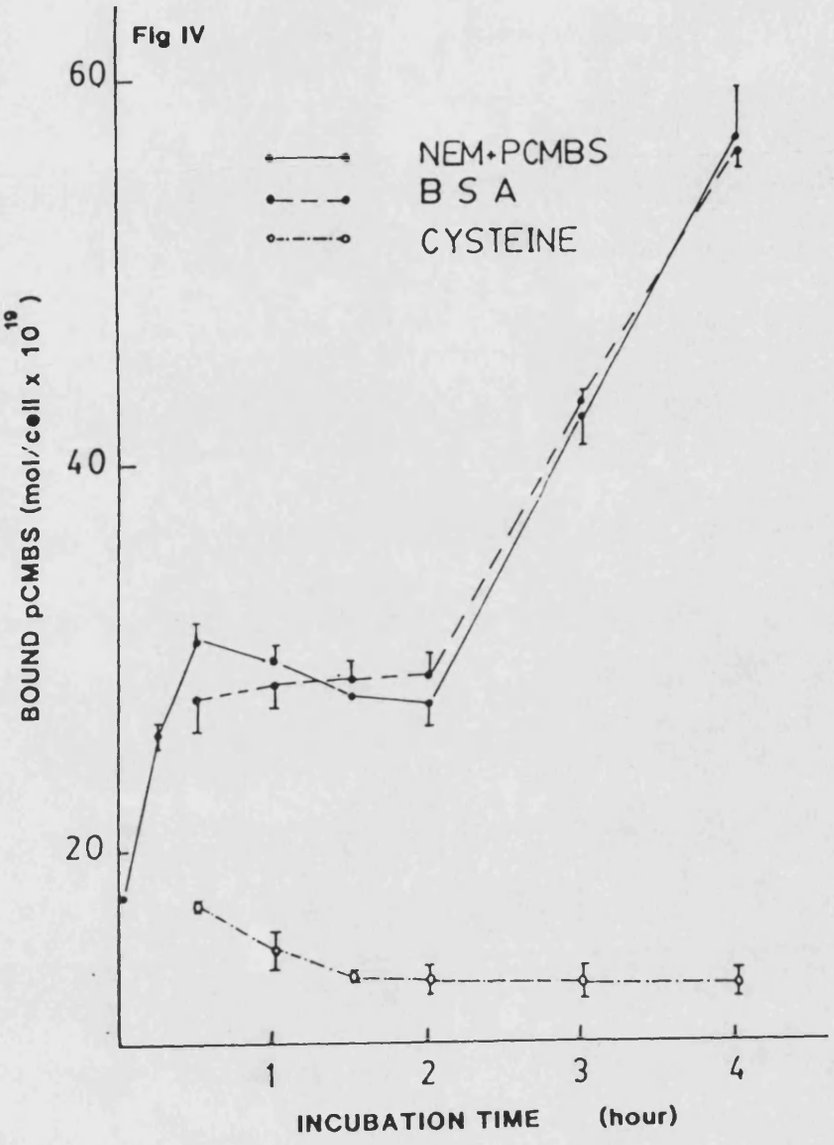


Fig IV
60
40
20
NEM+PCMBS
B S A
CYSTEINE
BOUND pCMBS (mol/cell x 10^19)
1
2
3
4
INCUBATION TIME (hour)

The results of the addition of cysteine suggest that the high binding capacity observed in the case of NEM + pCMBS or even after addition of BSA could be due to the binding of the labelled pCMBS to the NEM-insensitive sulphydryl groups and occupying in this case the internal and not the outer surface of the erythrocyte membrane. Another possible explanation is that NEM binds to the external -SH groups and prevents pCMBS from getting to them.

Figure V shows the effects of treatment with 1 mM thiolacetic acid. This compound reacts with the double bonded compounds in the cell membrane. The thiolacetic acid was incubated for 1 hour at 15°C pH 7.4 with the unreacted material being removed by washing. This was then followed by the addition of labelled pCMBS. In the same way as the previous experiment, the reversal effects of 10 mM cysteine and 6×10^{-4} M BSA were investigated 30 minutes post ^{203}Hg pCMBS addition.

The binding curve of ^{203}Hg-pCMBS showed an initial jump amounting to 22.6×10^{-19} mol cell^{-1} which is completed in a few minutes, followed by slow binding to a maximum amount of 30.6×10^{-19} mol cell^{-1} at 2 hours. A slower buildup of the agent was recorded during the next 24 hours amounting 36.6×10^{-19} mol cell^{-1}.

BSA initially desorbed 6×10^{-19} mol cell^{-1}, then showed a very slow buildup of the agent during the next 24 hours amounting to 27.6×10^{-19} mol cell^{-1}.

Cysteine resulted in desorption of 10 x 10^{-19} mol $cell^{-1}$ followed by slow desorption over the next 24 hours amounting to 12.6 x 10^{-19} mol $cell^{-1}$. It is probable that this pCMBS had entered the cell and became bound to the haemoglobin sulphydryl groups.

A one mM thiolacetic acid was incubated for one hour with eel erythrocytes suspended in saline phosphate solution at 25°C buffered at pH 7.4, followed by the addition of labelled pCMBS. The data is graphically illustrated in figure VI.

The binding curve showed an initial rapid uptake of 21 x 10^{-19} mol $cell^{-1}$ in the first five minutes. A build up of agent to a maximum binding of 79 x 10^{-19} mol $cell^{-1}$ was recorded at 5.5 hours, followed by a slow binding through the next 24 hours amounting 94 x 10^{-19} mol $cell^{-1}$. This high amount of the binding of pCMBS may be attributed to the new thiol groups added by TAA to eel erythrocyte membrane component.

BSA was added 30 minutes post pCMBS treatment and resulted in desorption of 7. x 10^{-19} mol $cell^{-1}$ from the outer surface of the membrane, followed by a slow buildup of the pCMBS to a maximum binding of 48 x 10^{-19} mol $cell^{-1}$ which could be attributed to reaction with the inner surface -SH groups.

The addition of cysteine resulted in desorption of 11 x 10^{-19} mol $cell^{-1}$ followed by a slow desorption of pCMBS from

Fig V and VI

Binding of ^{203}Hg-pCMBS to eel erythrocyte membranes pretreated with thiolacetic acid and reversed by cysteine and BSA.

Fig V at 15°C

Fig VI at 25°C

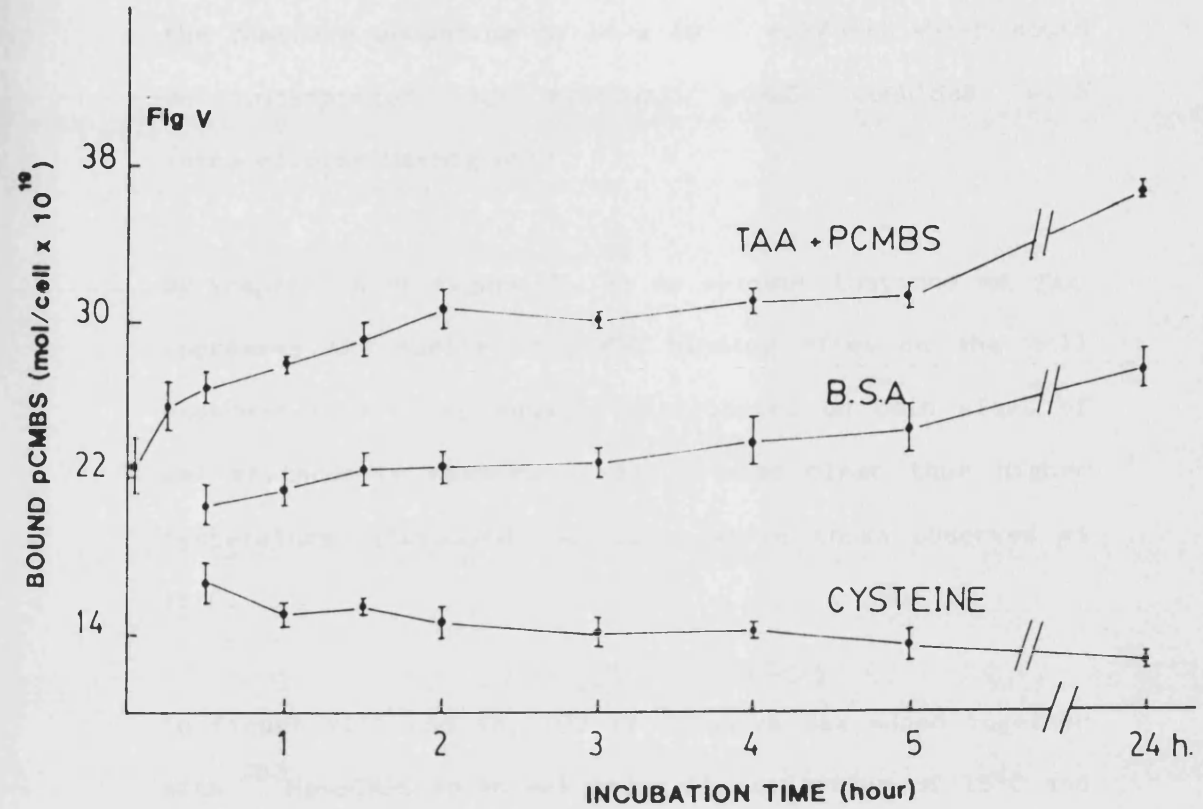


Fig V
38
30
22
14
BOUND pCMBS (mol/cell x 10^19)
TAA + PCMBS
B.S.A
CYSTEINE
1
2
3
4
5
24 h.
INCUBATION TIME (hour)

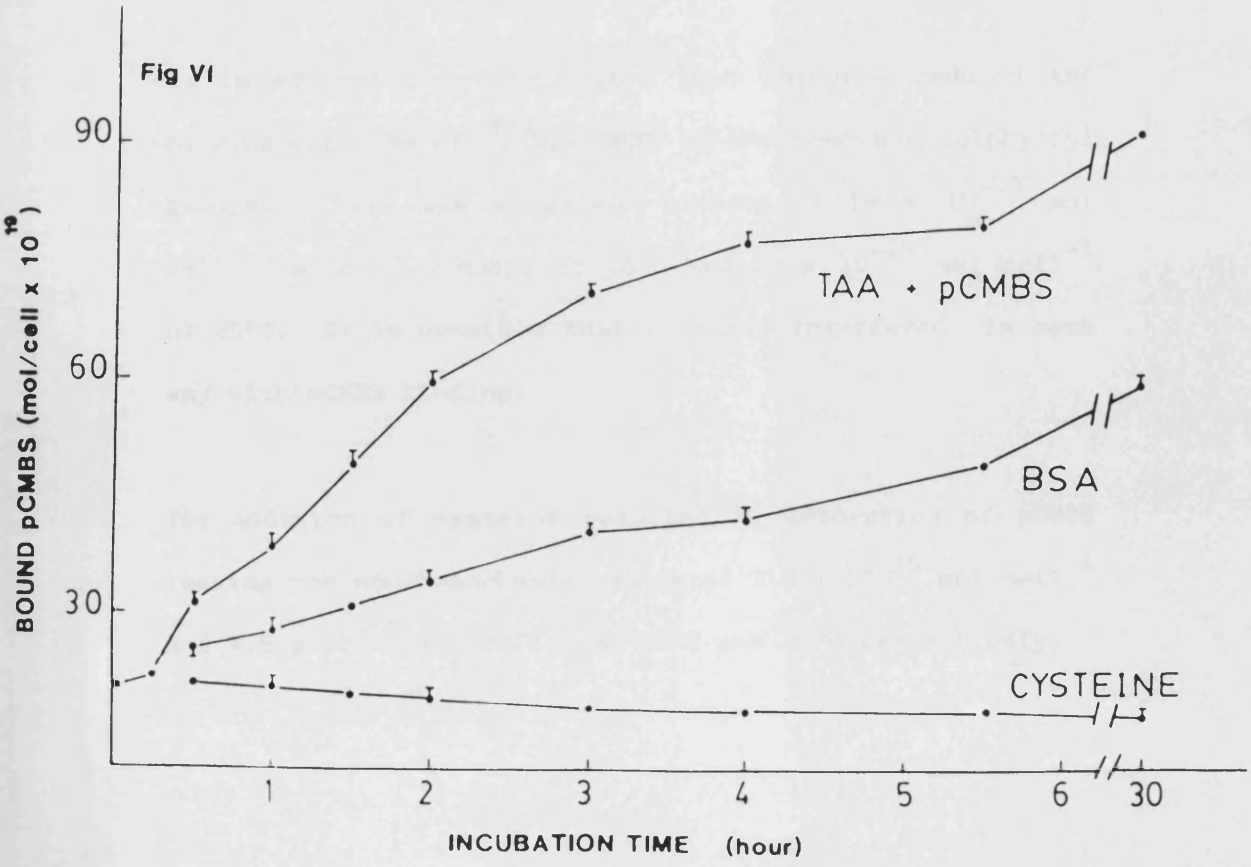


Fig VI
90
60
30
BOUND pCMBS (mol/cell x 10^19)
TAA + pCMBS
BSA
CYSTEINE
1
2
3
4
5
6
30
INCUBATION TIME (hour)

the membrane amounting to 14 x 10^{-19} mol/cell which could be interpreted as residual pCMBS combined with intracellular haemoglobin.

By inspection of figure VI, it is evident that one mM TAA increases the number of pCMBS binding sites on the cell membrane, they being equally distributed on both sides of eel erythrocyte membrane. It is also clear that higher temperatures increased the number above those observed at 15°C.

In figure VIII and IX, 100 mM thiourea was added together with ^{203}Hg-pCMBS to an eel red cell suspension of 15°C and 25°C respectively. In these experiments, since thiourea is a smaller molecule than cysteine it will pass through a channel which normally admits cysteine.

It is evident from the figure that thiourea reduced the binding capacity of ^{203}Hg-pCMBS to the membrane sulphydryl groups. There was a maximum binding of 19 x 10^{-19} mol cell $^{-1}$ after 3-5 hours at 15°C and 28 x 10^{-19} mol cell^{-1} at 25°C. It is possible that thiourea interfered in some way with pCMBS binding.

The addition of cysteine resulted in desorption of pCMBS leaving the non-desorbable amount of 3.8 x 10^{-19} mol cell^{-1} and 4.5 x 10^{-19} mol cell^{-1} at 15°C and 25°C respectively.

Fig VII and VIII

Binding of ^{203}Hg-pCMBS to eel erythrocyte membranes pretreated with 100 mM thiourea and reversal by cysteine.

Fig VII at 15°C

Fig VIII at 25°C

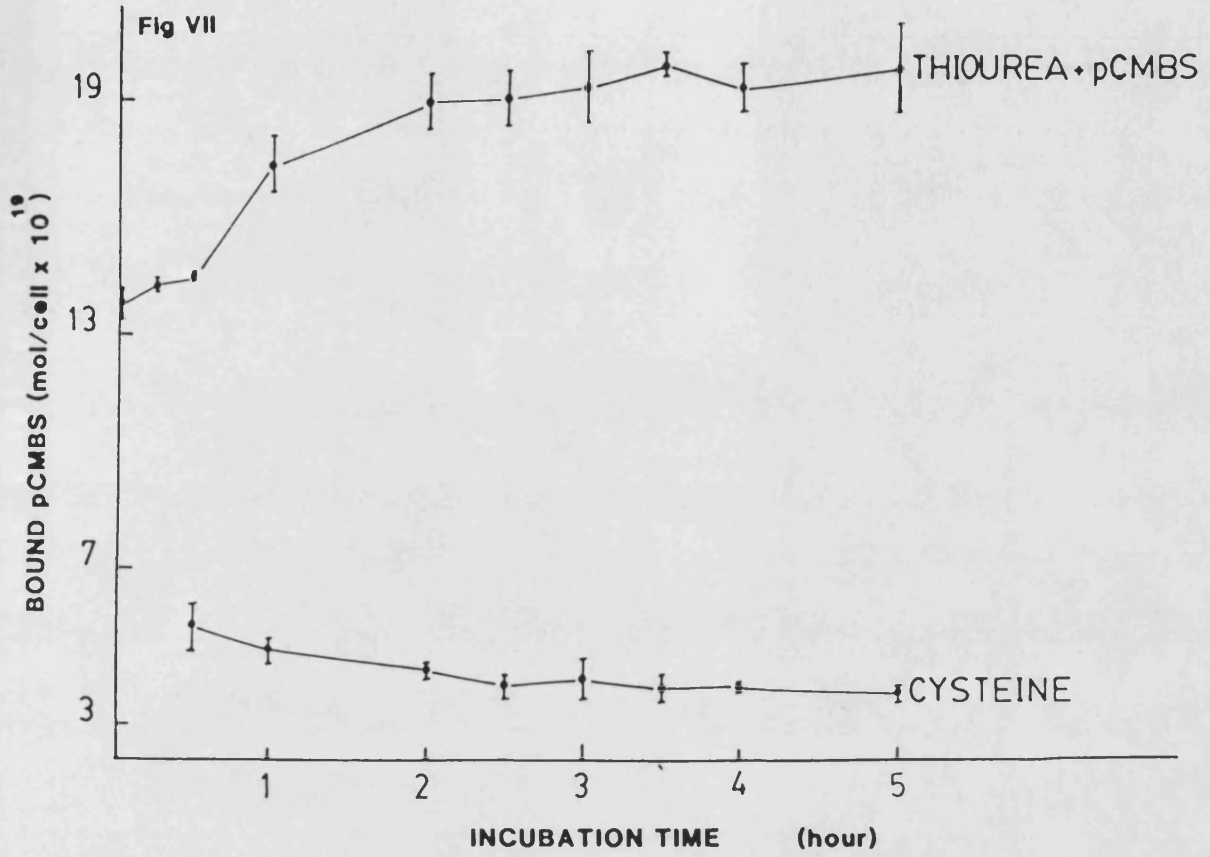


Fig VII
19
13
7
3
BOUND pCMBS (mol/cell x 10^19)
THIOUREA + pCMBS
CYSTEINE
1
2
3
4
5
INCUBATION TIME (hour)

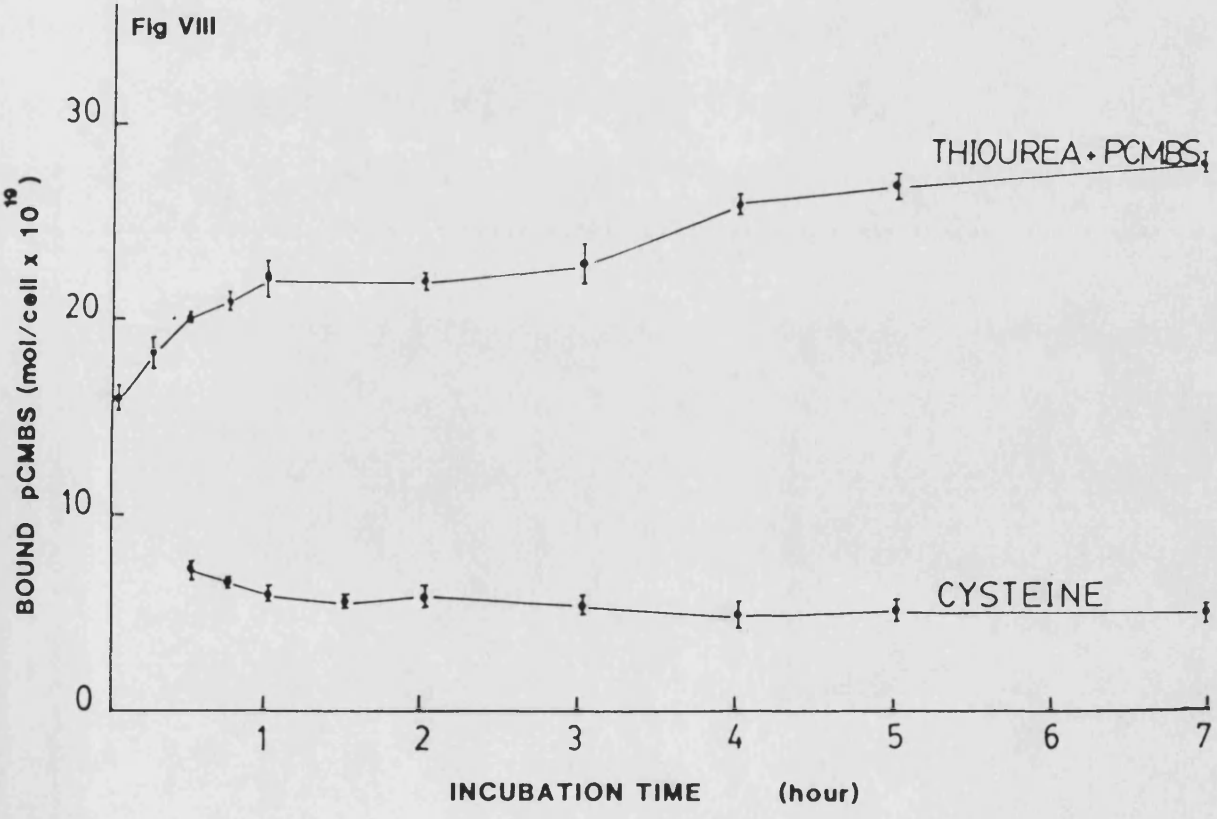


Fig VIII
30
20
10
0
BOUND pCMBS (mol/cell x 10^19)
THIOUREA + PCMBS
CYSTEINE
1
2
3
4
5
6
7
INCUBATION TIME (hour)

4.3.2 Human Erythrocytes

The binding curves of ^{203}Hg-pCMBS and its desorption at 15°C and 25°C pH 7.4 are graphically shown in figures IX and X respectively.

The binding curve of pCMBS to human erythrocyte membrane, can be divided into three component parts, (i) a very rapid initial binding completed within the first five minutes, amounting to 13.7 x 10^{-19} mol/cell and 2.8 x 10^{-19} mol/cell at 15°C and 25°C respectively, (ii) a further build up of agent completed in 30 minutes to reach a maximum binding of 17.6 x 10^{-19} mol/cell at 15°C and 49.47 x 10^{-19}/cell at 25°C, (iii) a continuous loss of pCMBS from the cell membranes during the 3 hours of the experiment to 9 x 10^{-19} mol/cell at 15°C and 31.6 x 10^{-19} mol/cell at 25°C. This loss has been attributed to the competition for the agent by soluble sulphydryl substances in the medium that are slowly released from the cells (Sutherland et al, 1967). It is evident also that the binding capacity depends on the temperature,for at 15°C there was less binding of pCMBS to that at 25°C.

The addition of Bovine Serum Albumin (BSA) 30 minutes post ^{203}Hg-pCMBS treatment resulted in a continuous desorption of the agent from the cell membrane to attain a final level of 7.5 x 10^{-19} mol $cell^{-1}$ and 24.3 x 10^{-19} mol $cell^{-1}$ at 15°C and 25°C respectively after five hours.

Fig IX and X

Binding of ^{203}Hg-pCMBS to human erythrocyte membrane -SH groups and reversal by cysteine and BSA.

Fig IX at 15°C

Fig X at 25°C

Fig IX

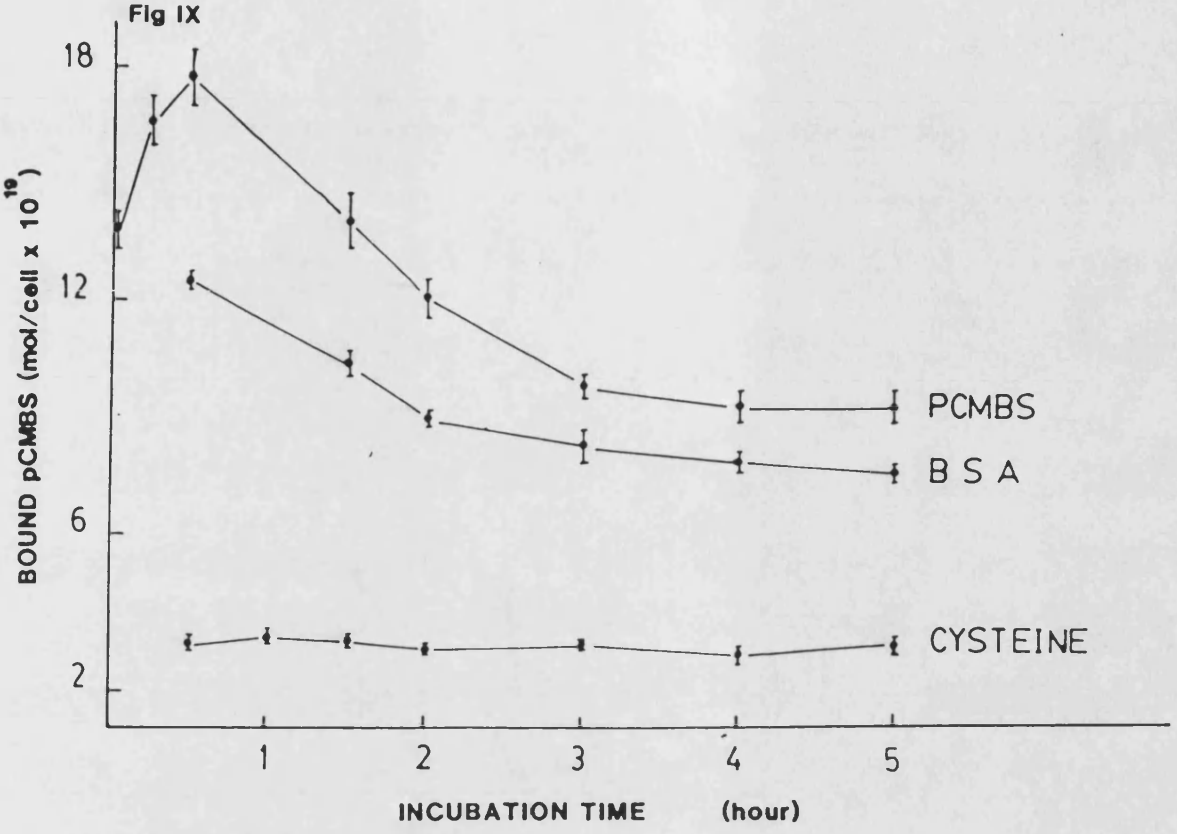
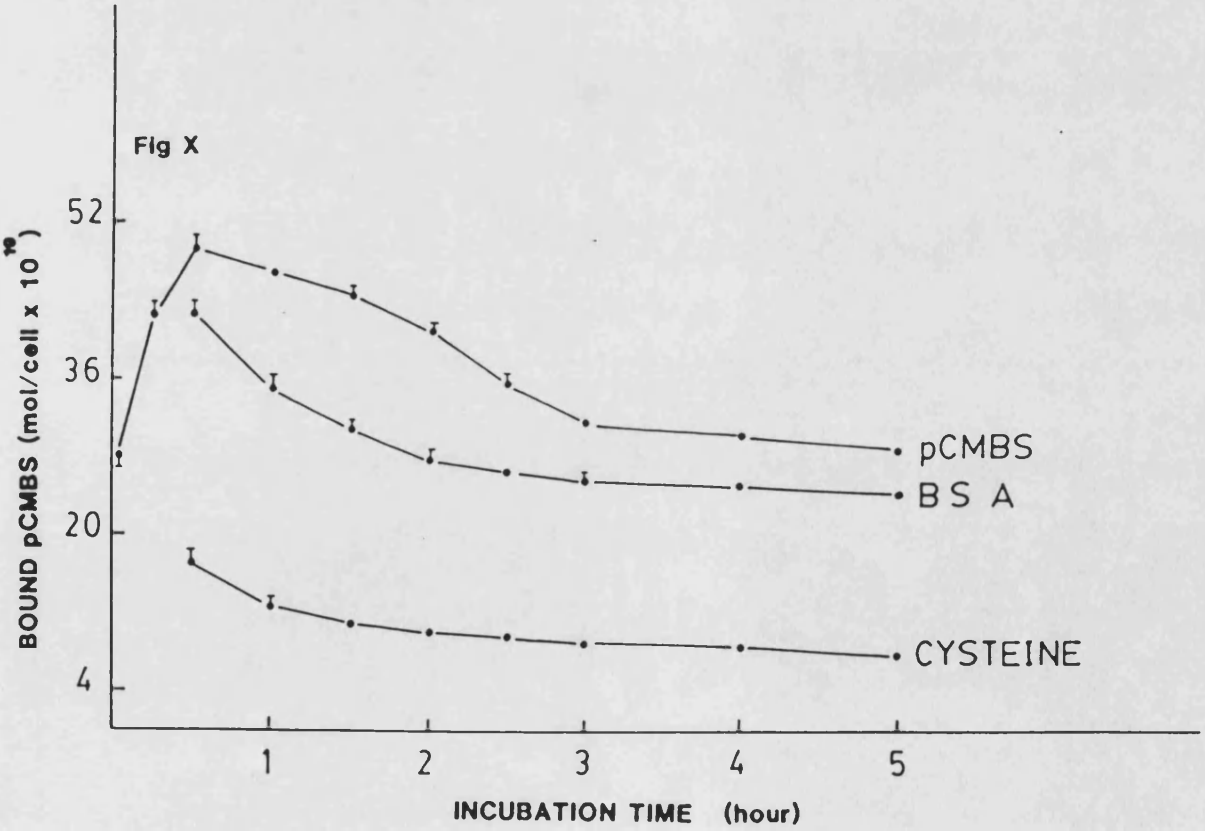

BOUND pCMBS (mol/cell x 10^19)
18
12
6
2
1
2
3
4
5
INCUBATION TIME (hour)
PCMBS
B S A
CYSTEINE

Fig X


BOUND pCMBS (mol/cell x 10^19)
52
36
20
4
1
2
3
4
5
INCUBATION TIME (hour)
pCMBS
B S A
CYSTEINE

Addition of cysteine after 30 minutes resulted in a rapid desorption of pCMBS reaching a level of 3.1×10^{-19} mol $cell^{-1}$ and 7.6×10^{-19} mol $cell^{-1}$ after 5 hours at 15°C and 25°C respectively.

Inspection of figures IX and X shows that about 32.4×10^{-19} mol $cell^{-1}$ (ie 80%) of pCMBS was desorbed by cysteine and 6.8×10^{-19} mol $cell^{-1}$ (ie 20%) by BSA at 25°C. The difference in the amount desorbed by BSA as compared to cysteine (1:5) should reflect the location of the sulphydryl sites on human erythrocyte membrane. 80% of SH groups are found on the cytoplasmic surface and only 20% on or very close to the outer surface of the red cell membrane. A similar distribution (1:5) was also observed at lower temperature.

It has been previously reported that for human cells, N-Ethylmaleimide can easily penetrate the membrane and combine with the cytoplasmic sulphydryl groups and block the subsequent reaction with pCMBS leaving the external groups free and sensitive to the pCMBS, (Rao, 1979).

In figure XI , 2 mM NEM was incubated with a human erythrocyte suspension for one hour at 25°C and pH 7.4 - followed by the addition of pCMBS. Bovine serum albumin and cysteine reversal effects were tested 30 minutes post pCMBS treatment. The binding curve showed a continuous buildup of pCMBS to a maximum binding of 97.8×10^{-19} mol $cell^{-1}$ after 4 hours, followed by a continuous desorption

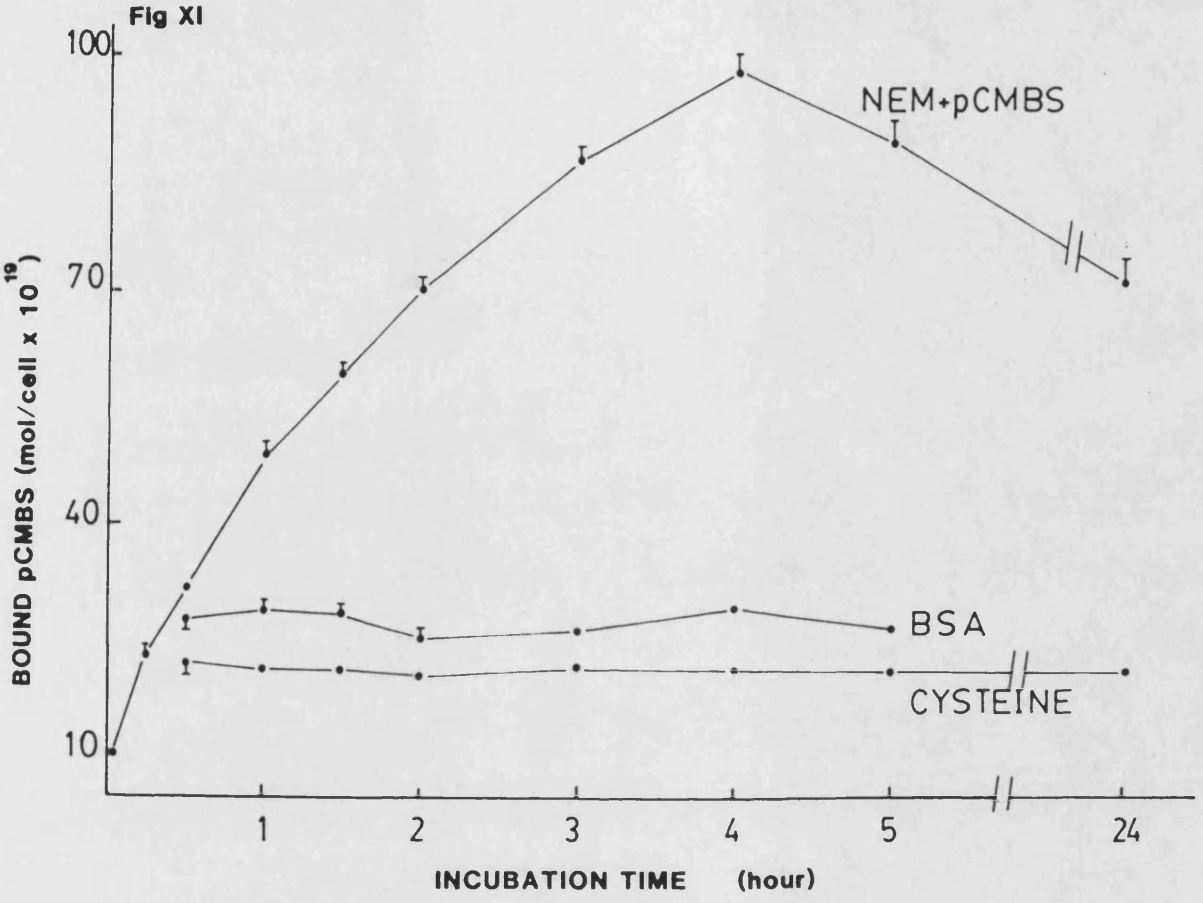


Fig XI Binding of ^{203}Hg-pCMBS to NEM-treated human erythrocyte membranes and reversal by cysteine and BSA at 25°C.

of the agent reaching 72 x 10^{-19} mol $cell^{-1}$ after 24 hours.

Addition of BSA and cysteine resulted in desorption of pCMBS from the cell membranes followed by constant binding levels throughout the experimental period.

Since NEM was added to the cell membrane, the high pCMBS uptake may be attributed to the external sulphydryl groups which appear more sensitive to the agent than in pCMBS only treated cells. This was simply confirmed by addition of BSA and cysteine which desorbed more or less equal amount of the pCMBS from the membranes throughout the experiment.

In figure XII thiolacetic acid (1mM) was incubated with a human erythrocyte suspension at 25°C and pH 7.4 for one hour. Before addition of pCMBS the TAA was washed out of the cells. Thiolacetic acid was removed because it is a small sulphydryl molecule and would easily combine with pCMBS if added along with it.

It is evident from the graph that TAA interfered with pCMBS uptake showing a slow buildup of agent to a maximum binding of 31.6 x 10^{-19} mol $cell^{-1}$ at 4 hours, followed by desorption of the agent from the cell membranes to a final level of 22.8 x 10^{-19} mol $cell^{-1}$ after 30 hours.

Addition of cysteine and BSA, 30 minutes post pCMBS showed a desorption of the agent from cell membranes.

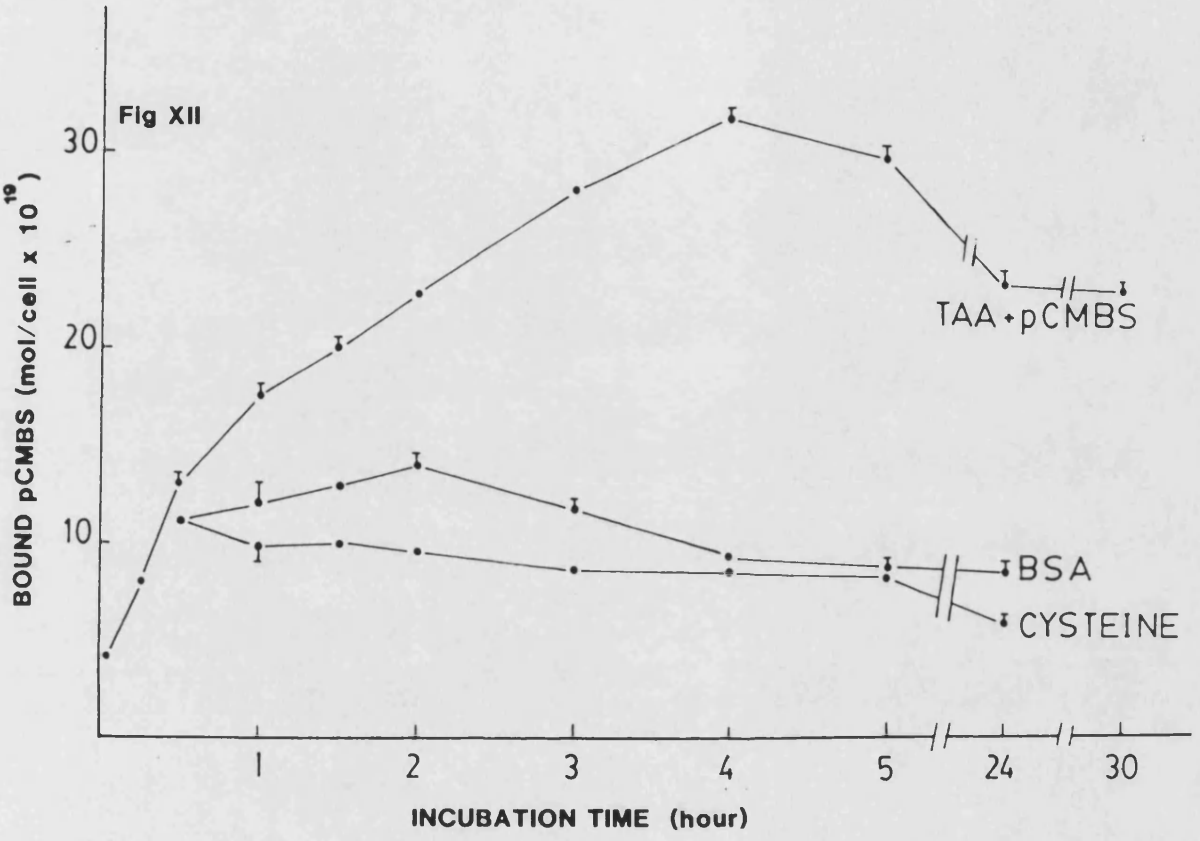


Fig XII Binding of ^{203}Hg-pCMBS to thiolacetic acid treated human erythrocyte membranes, and reversal by cysteine and BSA at 25°C

Thus TAA appeared to prevent pCMBS from reaching the internal SH groups and interfered with pCMBS binding to the external -SH sites on the erythrocyte membranes.

In figure XIII, pCMBS was added along with thiourea (100 mM) to the cell suspension at 25°C and pH 7.4 followed by addition of 10 mM cysteine, 30 minutes post pCMBS.

Thiourea inhibits the maximum binding capacity to 22 x 10^{-19} mol $cell^{-1}$ which is reached after 90 minutes, a much longer period than the pCMBS only treated erythrocytes, (30 minutes). This is then followed by a continuous slow desorption attaining a value of 13.4 x 10^{-19} mol $cell^{-1}$ after 24 hours.

Addition of cysteine resulted in a very rapid desorption of pCMBS from cell membranes leaving 3.4 x 10^{-19} mol $cell^{-1}$ after six hours as a non-desorbable level. Thiourea does not interfere with the cysteine reversal effect but does interfere with pCMBS uptake.

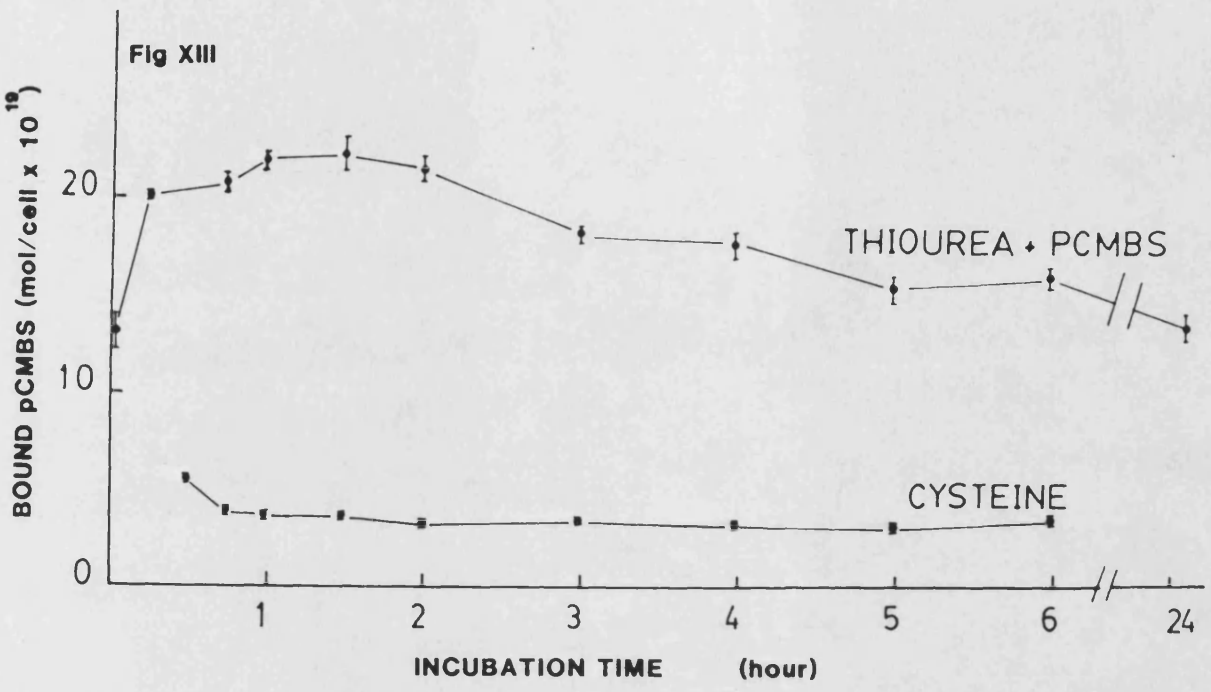


Fig XIII Binding of ^{203}Hg-pCMBS to thiourea-treated human erythrocyte membrane and reversal by cysteine at 25°C.

4.3.3 The total number of Sulphydryl Groups in Intact Cells, Cell membranes and Haemoglobin in Eel and Human Erythrocytes

Tables 1 & 2 show that the maximum number of binding sites for pCMBS on intact eel and human erythrocytes are the same - approximately 3.8×10^{-18} mol $cell^{-1}$. The difference between the cells is that the agent (pCMBS) desorbs from the human red cells on incubation, while, on eel erythrocytes the level of pCMBS binding remains constant.

The addition of 10 mM cysteine at varying times post-pCMBS treatment resulted in desorption of the agent from the intact human erythrocytes to leave a constant level of pCMBS independent of time of addition. In eel erythrocytes, the amount of non-desorbable residual pCMBS varies with the time of cysteine addition; a high level being left with the late cysteine addition. The level of this residual pCMBS is significantly ($p < 0.05$) higher ($1.77 \times 10^{-18} \pm 0.073$ mol $cell^{-1}$) in human than in eel intact erythrocytes ($1.24 \times 10^{-18} \pm 0.18$ mol $cell^{-1}$).

The addition of pCMBS to cell membranes free of haemoglobin showed that the number of binding sites for pCMBS in eel are considerably greater than for human cell membranes (tables 1 and 2) which may be attributed to the nuclear fragments.

The addition of cysteine to cell membranes from human and eel erythrocytes at different time intervals post pCMBS

treatment always resulted in desorption of large amount of the reagent to leave approximately equal amounts of the residual pCMBS. The final levels attained were found, unlike intact cells, to be time-dependent of cysteine addition.

The uptake of pCMBS (tables 1 & 2) by haemoglobin is significantly higher for human than for eel cells and the addition of cysteine causes desorption of about 70% of pCMBS bound to human haemoglobin and about 60% to eel haemoglobin. In most cases the residual pCMBS is independent of the time of cysteine addition to haemoglobin.

The percentage of ^{203}Hg-pCMBS bound to the intact erythrocytes was found to be 3.5 to 4% of the total sulphydryl groups of both human and eel erythrocytes. The binding to sulphydryl groups of haemoglobin was found to be 83% and 67% of the total cellular sulphydryl groups in human and eel erythrocytes respectively. The cell membrane (free of haemoglobin) constitutes about 17.5% and 33% of the total number of sulphydryl groups in human and eel erythrocytes respectively.

Table 1: Levels of pCMBS bound to eel erythrocytes

(Mol pCMBS $cell^{-1}$ x 10^{-18})

Sample	Time / Treatment	45 min	75 min	150min
Intact Cell	pCMBS	3.76 ±0.15	4.05 ±0.25	4.071 ±0.13
	+ Cysteine	1.018 ±0.15	1.237 ±0.17	1.457 ±0.008
Cell Membranes	pCMBS	31.50 ± 0.037	32.38 ± 0.08	35.20 ± 0.067
	Cysteine	2.23 ± 0.005	1.41 ±0.03	1.61 ±0.016
Haemoglobin	pCMBS	67.398 ± 2.16	66.614 ± 1.56	68.48 ± 0.83
	Cysteine	27.55 ± 0.64	26.37 ± 0.22	26.22 ± 0.58

*pCMBS concentration is 6 x 10^{-5} M

Table 2: Levels of pCMBS bound to human erythrocytes

(Mol pCMBS $cell^{-1}$ x 10^{-18})

Sample	Time / Treatment	45 min	75 min	150min
Intact Cell	pCMBS	3.25 ±0.40	3.76 ±0.38	2.91 ±0.14
	+ Cysteine	1.72 ±0.16	1.88 ±0.17	1.73 ±0.008
Cell Membranes	pCMBS	17.17 ± 3.6	14.65 ± 2.9	19.66 ± 0.92
	Cysteine	1.93 ± 3.8	2.989 ±0.27	1.948 ±0.97
Haemoglobin	pCMBS	80.67 ± 1.54	82.446 ± 0.55	84.70 ± 1.41
	Cysteine	21.02 ± 3.12	25.526 ± 4.60	12.97 ± 0.77

*pCMBS concentration is 6 x 10^{-5} M

4.4 DISCUSSION

Localization of Membrane Sulphydryl groups

The present study demonstrates clearly that the nature of the binding of the organic mercurial compound, pCMBS, to intact eel and human erythrocytes is significantly different. The pattern of pCMBS binding to intact human membranes confirms the previous works of Sutherland et al (1967) and Rega et al (1967). The data show that maximum binding was reached 30 minutes post ^{203}Hg-pCMBS treatment followed by a continuous desorption of the agent due to the continuous release of a sulphydryl containing substance (Sutherland et al (1967) and Weed et al (1962)). In contrast, the ^{203}Hg-pCMBS binding to eel intact erythrocytes is immediate and is not lost over the experimental period, suggesting that no -SH groups containing substances are released from eel cells.

The binding experiments also show that the sulphydryl groups of the human erythrocyte membrane are quite different in their distribution from that of the eel. The binding of ^{203}Hg-pCMBS and its removal by the non-penetrating molecule bovine serum albumin (BSA) and the readily penetrating cysteine reveal that the sulphydryl groups on the eel red cell membrane are equally divided on either sides of the erythrocyte membranes whereas for human cells about 80% of the pCMBS sulphydryl reactive groups are located on the inner surface (cytoplasmic side) and the

rest near the outer surface. The human data are in agreement with that previously reported by Rao and Reithermeier (1979). These authors discovered five -SH groups on the cytoplasmic side of the cell membrane (in band 3) reacting with NEM and pCMBS, while Ramajeesingh et al (1980) and Solomon et al (1983) have reported an additional sixth-SH-group in band 3 of human erythrcotye membrane that reacts only with pCMBS and not with NEM. Solomon et al (1983) has argued that this sixth SH group is extracellular because stilbene inhibitors do not change the pCMBS influence on water transport, whereas they do prevent access of pCMBS to the intracellular SH groups.

Based on the interpretation of experimental data for the geographical location of the sulphydryl groups within human red cell membrane, it is possible to say that 50% of pCMBS reactive groups are located on or very near to the outer surface of eel red cell membrane and the other 50% found near its deep side (cytoplasmic surface). A schematic diagram (in figure E) shows an assumed location of SH groups in the eel erythrocyte membrane.

Fig E

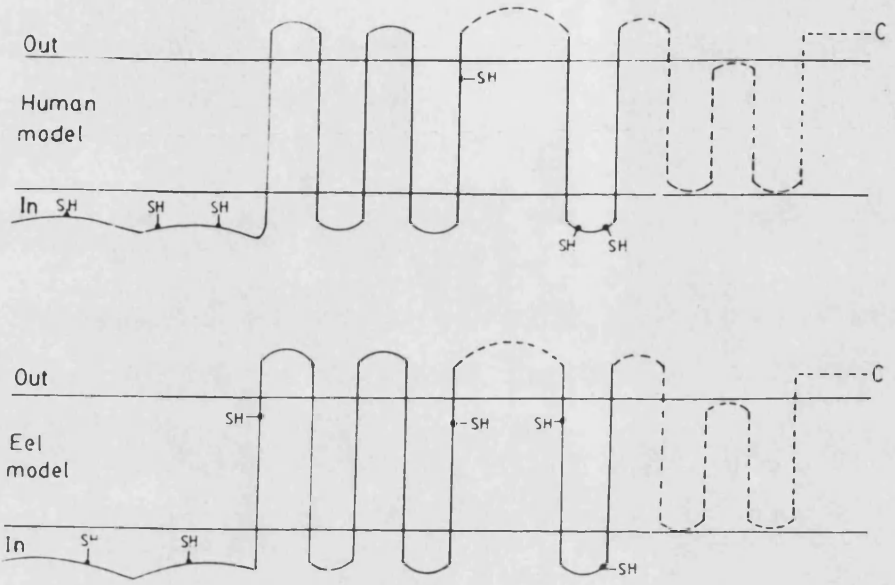


Figure E **Schematic diagram showing a possible location of SH groups in eel erythrocyte membrane based on the human model. The human model was taken from Rao and Reithmeier (1979) and Solomon** et al **(1983).** In both models, the number of loops is purely arbitrary. In the eel model the number of -SH groups is arbitrary.

Not only are the locations of SH groups different in eel and human but also they are different in their reactivity towards the specific sulphydryl reagent NEM. In NEM-treated human erythrocytes, the ^{203}Hg-pCMBS binds to the external SH groups that are insensitive to NEM. This is confirmed by the observation (Fig XI) that BSA and cysteine desorb the same amount of the labelled pCMBS. In the case of the eel erythrocyte, the ^{203}Hg-pCMBS binds to the intracellular SH groups in NEM-treated eel erythrocytes, implying that, unlike human cells, these groups are NEM insensitive. This effect was clearly shown by the inability of BSA to desorb pCMBS from its deep binding sites. These results suggest that the NEM-insensitive SH groups, which are involved in water transport (see section 3), are present extracellularly in the case of human and intracellularly in case of eel erythrocyte membrane.

The observation that there is an increasing number of SH groups that bind pCMBS when cells are treated with NEM may be explained by the fact that NEM inhibits the production and efflux of glutathione or glutathione based material, from intact erythrocytes, (Jacob and Jandl , 1962). Shapiro et al (1970), have demonstrated that the reaction between pCMBS and GSH is reversible in the presence of haemoglobin but irreversible between NEM and GSH under the same conditions.

The fact that erythrocyte membranes contain multiple classes of sulphydryl groups (Sutherland et al, 1967) was further developed by Shapiro et al (1970) and characterized as reactive, partially reactive and masked groups. Reithmeier and Rao (1979) have attributed the unreactive nature of the membrane SH group to the conformational change that occurs upon cross-linking. It is possible therefore that, the cross-linking that occurs between pCMBS and the SH groups on the membrane (Band 3) would preclude the formation of other bonds by virtue of their high affinity. It is likely that the number of intersubunit bonds involved in cross-linking will depend on the sulphydryl reagent used. This idea is suggested in this study by the obvious difference in the number of SH groups reactive to NEM or pCMBS alone and to NEM + pCMBS combination. Treatment with one sulphydryl reagent could increase or decrease the number of SH group available for interaction.

The sulphydryl containing compound, thiolacetic acid (CH_3 CO SH, RMM 76.12) is a reagent that reacts with unsaturated compounds (Brown et al, 1951) but has no effect on the pattern of pCMBS binding or its reversal by BSA and cysteine in eel erythrocytes. In human cells, thiolacetic acid interferes and prevents the access of the labelled pCMBS to intracellular membrane SH groups, a fact clearly shown by the same amount desorbed by BSA and cysteine. (Fig XII).

Thiourea is equally a small molecule (RMM 76.12) which readily penetrates the red cell. The addition of thiourea together with ^{203}Hg-pCMBS considerably interferes with the binding of the reagent to both eel and human. This observation would explain the finding of Chasan et al (1984) who observed that the addition of 100 mM thiourea prevents the inhibitory action of pCMBS on the osmotic water transport across human erythrocyte membrane. Thiourea prevents pCMBS from reaching its binding sites.

The sulphydryl groups vary considerably in their number and distribution throughout the whole erythrocyte. Less than 5% was found on the membranes of both intact human and eel erythrocyte, by far the greatest amount being found on the haemoglobin when levels of 83% and 67% were recorded for human and eel respectively, while only 17% and 33% was recorded in haemoglobin free stroma of human and eel respectively. These percentages are in agreement with that of Weed et al (1962) and Vansteveninck et al (1965) of less than 5% in the intact cells, 90% in haemoglobin and 22-25% for stromal phase. These data indicate that the control of water transport is associated with that small fraction located on the membrane of the intact cell.

Chapter 5

Intracellular Water and ion levels in normal and pCMBS treated cells

5.1 INTRODUCTION

The maintenance of selective permeability of the red cell membrane to water and ions requires the integrity of certain sulphydryl groups in the membrane. The oxidation of these groups with the formation of disulphide bonds (Sutherland and Pihl, 1968) increases cation leakage and can eventually result in lysis.

The reaction of membrane sulphydryl groups with a variety of specific reagents, such as inorganic and organic mercurial compounds, results in a loss of potassium, a gain of sodium and haemolysis (Sutherland et al, 1967). These effects are produced after a delay period of 2 hours post mercury exposure and are with the exception of lysis, reversible. Based on the dependence of the delay period on both temperature and mercurial concentration, it has been suggested that the critical sulphydryl groups for ion and water balance are isolated from the external medium and form part of an interior compartment of the membrane (Sutherland et al, 1967). On this basis, rapidly penetrating lipid soluble mercurials (eg pCMB) would be expected to enhance the cation permeability faster and to a greater extent than the less permeable pCMBS. Surprisingly, the opposite has been found (Knauf and Rothstein (1971) and Shapiro et al, 1970), the fast permeable compound p-chloromercuribenzoate is considerably less effective than an equal concentration of pCMBS. Furthermore, non-ionic mercurials such as 1-bromomercuri-2-hydroxypropane (BMHP)

that enter even more rapidly than pCMB are without effect on cation permeability (Shapiro et al, 1970). The interpretation of such an inverse relationship between permeation rate and effects requires a reappraisal on the location of the sensitive sulphydryl sites, the pathways by which the sulphydryl reagents penetrate, and the protective effects of haemoglobin in binding the agents as they reach the cytoplasmic surface.

To circumvent such complications and to avoid possible differences in the reactivity of different chemical probes, Sutherland et al (1967a) studied only the reaction of the slowly penetrating organic mercurial compound, pCMBS, with intact human erythrocytes, and conclusively demonstrated its binding, within the interior of the membrane with subsequent alterations in both Na^+ and K^+ permeability. The increased cation permeability and accompanying haemolysis were observed only after a lag period of two hours.

Sutherland et al (1967b) also studied the relationship between the rate of binding of the agent and its final effect and showed that a small but rapid initial binding does not affect cation permeability but the subsequent slower uptake is associated with increased leakage of K^+ and Na^+. The observed natural recovery at 25°C (but not at 2°C) is associated with desorption of about half of the pCMBS due to competition by soluble thiol substances released into the medium from the cells.

The (Na^+ + K^+) activated ATPase in the erythrocyte membrane is inhibited by such sulphydryl reacting compounds as N-ethylmaleimide (Weed and Berg (1963) and Tosteson (1966)), chlormerodrin (Weed and Bery, 1963) and pCMB (Skou, 1963). Since both (Na^+ + K^+) ATPase activity and normal cation permeability appear to be dependent on -SH groups located within the membrane, the investigations of Rega et al (1967) comparing the action of pCMBS on both the passive and active transport of cations are important. Apparently pCMBS has two opposing effects on the influx of Rb^+ into human red cells - the ouabain-insensitive component indicative of passive permeability, is increased, whereas the ouabain-sensitive or active transport component is inhibited. The workers also demonstrated that the increased passive permeability is paralleled by the action of pCMBS on net K^+ efflux into a K^+ free medium, the ouabain-insensitive Rb^+ influx or K^+ efflux being increased by 70% and 150% respectively over the first hour. The efflux of K^+ at 25°C can in fact be increased by over 600% (Sutherland et al, 1967 and Rega et al, 1967). These latter authors also reported that the inhibition of active transport of Rb^+ is paralleled by the inhibitory action of pCMBS on net Na^+ efflux from Na^+-rich cells. All these effects are rapidly reversed by the penetrating sulphydryl compound, cysteine, and considerably more slowly by the nonpenetrating sulphydryl compounds, ovalbumin (Rega et al, 1967) and bovine serum albumin (Sutherland et al, 1967).

The fact that the -SH groups responsible for the changes in cation permeability are more accessible from the inside face of the membrane was investigated by addition of pCMBS to either the external or to the cytoplasmic face of haemoglobin-free red cell membranes. This was achieved by preparing resealed right-side-out or inside-out membrane vesicles derived from human red cells, (Grinstein and Rothstein, 1978), loading them with radioactive Rb^+ (which serves as a K^+ analogue for both active and passive fluxes in red cells (Rega et al, 1967) and then measuring the effects of pCMBS on the efflux of the isotope. The results of Grinstein and Rothstein (1978) clearly showed that identical concentrations of pCMBS produce larger and faster increases in the efflux of Rb^+ in inside-out than in right-side-out vesicles. Moreover certain concentrations of pCMBS which cause a substantial enhancement of the efflux from inside-out vesicles produced little or no change in the right-side out vesicles within the period of time studied. It was also shown that at 0°C the high concentration of pCMBS (500 μ M) had no effect on right-side out vesicles for 20min with minimal effect thereafter, whereas the response for inside out vesicles was substantial (50% loss of Rb^+ in 20 min) suggesting that the SH groups involved in cation permeability are more accessible to, but not on, the cytoplasmic surface of the membrane and that the pCMBS must traverse a diffusion barrier to reach them.

pCMBS is highly specific for sulphydryl groups confirming that its effects on permeability are mediated by membrane proteins (Rothstein, 1970). In an attempt to identify these particular proteins, a selective removal of some intrinsic proteins was accomplished by means of an alkaline extraction (NaOH and EDTA) in a low ionic strength medium (Steck et al, 1976). This treatment removed bands 1, 2 and 5 and considerably depleted band 6. ^{86}Rb efflux was measured in such depleted inside-out vesicles at 21°C by Grinstein and Rothstein (1978) who found that the mercurial pCMBS immediately increased the efflux of ^{86}Rb several fold suggesting that the permeability response can be attributed to intrinsic proteins not removed by alkaline extraction.

The major intrinsic protein of the red cell membrane is the band 3 (see chapter 4 p 111) which contains 5 sulphydryl groups on the cytoplasmic surface. One of these can form intermolecular crosslinks in the presence of a O-phenanthroline-Cu SO_4 complex (Steck, 1972 and Steck and Kant, 1974) and may be involved in cation permeability regulation. Under the crosslinking treatment most of band 3 molecules are dimerized (Steck, 1972) and using this system, Grinstein and Rothstein (1978) showed that such treatment of band 3 had no effect on cation fluxes or on the influence of pCMBS.

A purification of a component having active -SH groups was achieved by combining alkaline extraction with the use of

proteolytic enzymes to digest portions of the protein exposed on the outer and inner surface of the membrane. Grinstein and Rothstein (1978) treated the intact cells with chymotrypsin before their membranes were converted to inside-out vesicles. The latter were then treated with trypsin and extracted with an alkaline, low ionic strength buffer solution. (As previously mentioned in chapter 4 the major component is a 17 KDa segment derived from band 3 after loss of a 35 KDa segment from the outside, and 42 KDa segment from the inside). Even after this treatment, a substantial effect from pCMBS was still observed. The response was delayed and slightly smaller in these digested vesicles than in intact ones, but more than 95% of Rb^+ was liberated from them by pCMBS. This suggests that peptides remaining in the membrane after proteolysis and alkaline extraction can respond to sulphydryl agents with a change in Rb^+ permeability. Grinstein and Rothstein (1978) suggested that the 17 KDa transmembrane polypeptide is a logical candidate for cation permeability and could provide an aqueous protein channel through which the Rb^+ might flow.

The similar effects of pCMB, pCMBS and chloromerodrin on potassium loss and sodium accumulation were observed by Shapiro et al (1970) and suggests that all these agents bind to the sulphydryl groups in the membrane that are involved in the maintenance of a sodium-potassium barrier. These sulphydryls are accessible to glutathione, which does not enter the cells, but which removes pCMB, pCMBS and chloromerodrin and restores the $Na^+ - K^+$ barrier.

The Li_i - Na_o countertransport system of human red cell was not inhibited by pCMBS within one hour of exposure (Hassa et al, 1975), but Levy and Livne (1984) observed inhibition after 24 hours incubation with pCMBS suggesting that the involved sensitive -SH groups are not externally exposed. It was also shown, however, that rapid (within seconds) inhibition of Li_i - Na_o countertransport activity occurred with the sulphydryl reagents N-ethylmaleimide (NEM), iodoacetate and iodoacetamide. Diamide, an SH-oxidizing agent that crosslinks spectrin and purturbs the asymmetric phospholipid arrangement (Haest et al, 1978), also inhibits the Li_i - Na_o countertransport. Preincubation with NEM prevented the effect of diamide on the membrane (Haest et al, 1978).

The Na^+/H^+ exchanger, involved in the regulation of cytoplasmic pH and cellular volume in a variety of cells (Grinstein et al, 1985) shows a significant inhibition (64%) in lymphocytes by diethylpyrocarbonate (a histidine-specific reagent) and by N-ethylmaleimide (a sulphydryl group reagent). Both pCMB + pCMBS also produce Na^+/H^+ countertransport inhibition but to a lesser degree (30%). A smilar reactive, but nonpermeating sulphydryl agent, glutathione-maleimide, failed to inhibit $Na-H^+$ exchange, suggesting that the chemically reactive groups of the Na^+/H^+ exchanger of lymphogytes, like those of erythrocytes, have a limited exposure to the extracellular medium and that the internally located sulphydryl groups

are critical for the cation exchange activity.

The early work of Jacob and Jandl, (1962a) demonstrated that pCMB induces an immediate and very pronounced swelling of the erythrocyte which was later shown to be due to the disruption of the cation gradient across the membrane (Levy and Livne, 1984). The influx of water led to spherocytosis and swelling of the cells resulting eventually in haemolysis (Jacob and Jandl , 1962a). A similar effect was observed with NEM but only after a lag period of 4 hrs. Initially, NEM caused cell shrinkage, but after 4 hours a gradual sphering and swelling occurred. Sutherland et al (1967) similarly demonstrated a cell volume increase despite the large losses of K^+ from pCMBS-treated human red cells. The K^+ loss was accompanied by Na^+ gain and was further confirmed by an experiment in which choline chloride was substituted for NaCl in the suspending medium resulting in a large decrease in cell volume. The sulphydryl groups which react with BMHP (1-bromomercuri-2-hydroxypropane) have not been implicated in sodium-potassium permeability, but are involved with some membrane permeability function, as haemolysis occurs not only in cells suspended in isotonic sodium chloride and choline chloride but also in lactose solution, (Shapiro, et al, 1970).

The observed haemolysis of cells subjected to pCMBS or pCMB can be prevented by the addition of cysteine, albumin, sucrose and interestingly by a solution containing not less

than 20 mM of Mg^{++} which in the latter case could reverse by 63% the amountof pCMBSinduced haemolysis, (Jacob and Jandl , 1962).

The interaction of pCMBS with membrane sulphydryl groups has no effect on anion permeability as measured by sulphate fluxes (Knauf and Rothstein, 1971a), while the drugs SITS (4-acetamido-4'-isothiocyano stilbene-2,2' disulphonic acid) reacts with membrane amino groups and decreases anion permeability. Because pCMBS is an anion, its uptake is considerably reduced in SITS-treated red cells (Knauf and Rothstein, 1971b) but its effect on cation permeability is not reduced. These data suggest that pCMBS enters the membrane by at least two channels, one sensitive and the other insensitive to SITS, with only the latter leading to the cation-controlling sulphydryl groups. Knauf and Rothstein (1971a) further observed that the substitution of phosphate or sulphate for chloride in the bathing medium results in an inhibition of pCMBS uptake via the SITS-insenstive pathway. These data are in accord with those reported recently by Passow (1986) who demonstrated that pCMBS, like other -SH reagents, has no direct assessable effect on anion transport in human red cells. Solomon and his associates (Lukacovic et al, 1984b and Yoon et al, 1984) however have shown that there exists some allosteric relationships between specific pCMBS binding sites (possibly the -SH group on 17 KDa of band 3) and stilbene disulphonate binding sites and have demonstrated that pCMBS inhibits the binding of DBDS (4-4'

dibensoamido-2-2'-disulphonic stilbene, a specific anion exchange inhibitor) to band 3 protein. DBDS does not affect water permeability in human red cells.

Other sulphydryl reagents such as NEM, iodoacetate or iodoacetamide not only do not affect water permeation but also do not interfere with DBDS binding. This observation prompted Solomon and his co-workers to propose that, perhaps, both water and anion transport are mediated by the band 3 protein. They derived further support for this view from the use of stilbene derivatives which, instead of the isothiocyanate groups present on DIDS, contain $HgCl_2$ residues and are thus capable of reaction with SH groups. This compound called 4-4' dichloromercuric -2,2,2',2' bistilbene tetrasulphonic acid (DCMBT) inhibited anion transport instantaneously and completely whereas water was only slowly and partially inhibited (15-20%) (Yoon et al, 1984). Treatment of red cells with DIDS inhibited the effect of DCMBT on water transport. Yoon et al (1984) therefore suggested that the site of control of water transport is distinct from stilbene disulphonate binding sites but is nevertheless located in the same channel that serves anion transport.

Because mercurials enhance cation efflux, studies with liposomes containing purified band 3 protein treated with mercurial compounds (Lukacovic et al, 1984a) showed an enhancement of K^+ efflux suggesting that

band 3 may serve as a channel for anions, cations and water.

This chapter is devoted to a study of the ion and water permeabilities of mercurial treated eel cells in an effort to contrast the controlling mechanisms of the -SH groups with those of human cells.

5.2 MATERIALS AND METHODS

5.2.1 Materials

5.2.1.1 Erythrocytes

Red blood cells from eels and human subjects were collected and prepared as previously described in chapter 2.

For ion and water content determinations, the washed red cells were equilibrated overnight in the normal physiological saline (300-310 mosm Kg^{-1}) at 11-12°C to ensure a steady state condition with respect to cell volume and ion content. Because of the washing and suspension of cells in physiological saline, catecholamines, present in the plasma, would have been absent in these experiments and cells would have been in their lower steady transporting state (Bourne and Cossins, 1982). This procedure was necessary however to achieve reproducibility amongst cells sampled from animals over a protracted project of several seasons.

The haematocrit was kept low at 9% so that glucose was not significantly depleted during equilibration.

5.2.1.2 Chemicals

Highest purity grade chemicals were used throughout and non radioactive ones were purchased from Sigma Chemical

Company. These chemicals are:

a - pCMBS (see section 3)

b - Ouabain and furosemide at a concentration of 10^{-3} M.

c - cysteine (see section 3)

d - ^{14}C-Inulin (Amersham International)

5.2.2 Methods

Determination of water and ion contents of the Erythrocytes

5.2.2.1 Determination of water content

The method of Shawkat (1984) was adopted. Cell water content was estimated from differences between the wet and dry weight of an aliquot of cell suspension at haematocrit of 7-9%. Samples of blood were transferred to preweighed Eppendorf tubes and centrifuged for 10 minutes at 9000 g followed by aspiration. The resultant pellet was dried to constant weight at 80°C for 24h. Correction for trapped plasma volume was made routinely using labelled inulin (see 5.2.2.3.). The water content of the erythrocytes was expressed as ml H_2O per gm dry cell solid.

5.2.2.2 Determination of Cellular Ion Content

5.2.2.2.1 Na and K Ions

Cellular Na and K content was measured using emission spectroscopy (Jenway PFRI flame photometer) calibrated within the range of 0-10 mg l^{-1} for both ions. The dried cell pellets were extracted with distilled water, whirli-mixed, and Na^{+} and K^{+} analysis performed on the extract (Shawkat, 1984).

In all cases correction for ion concentration in the trapped medium was conducted and the ion content expressed as m mol/kg dcs (dry cell solid).

5.2.2.2.2 Chloride ion

Chloride concentration was measured in the extract of cell pellets using a coulombometric titration technique (CMT 10 chloride titrator). The instrument was standardised at 103 mM NaCl Kg^{-1} and gave readings with a reproducibility of $\pm$ 0.5% including a pipetting error of 0.2%. The chloride content was expressed as m mol/Kg dcs.

5.5.2.3 Determination of Trapped plasma Volume "E.C.V"

The method of Shawkat (1984) was followed. The determination of trapped medium volume (extracellular

volume) was conducted routinely with each experiment and performed by suspending the cells in a solution containing 185 KBq ^{14}C-inulin at a haematocrit 7-9%. At each sampling period a 40 μl aliquot of cell suspension was taken for liquid scintillation counting as was a 40 μl sample of the bathing medium. The activity of extracellular fluid was corrected for chemical quenching by the external standards method. The liquid scintillation fluid used was Opti Phase (Fisons/LKB) and the counter was a Rak Beta type 1217 liquid scintillation counter (LK13, Walkac, Finland).

Because of the large number of determinations carried out in this section of the work, a simple computer program in BASIC was written for a BBC microcomputer to speed the routine calculation for water and ion content of the cells. The programme is detailed in Appendix

5.2.2.4 Determination of percentage Haemolysis

The percent haemolysis values were obtained by estimating the haemoglobin concentration in the supernatant of sample compared to that from a 100% haemolysis sample. Concentrations were determined by the standard cyanomethe -moglobin method using Drabkin's reagent (Sigma Chemicals USA) (see Chapter 4).

Problem of Osmotically inactive (Bound) water in cells

The presence of osmotically inactive water in the cell has been debated over the last two decades (Hinke 1970 , Shawkat 1984), with no firm conclusions being reached. Working on the Barnacle muscle fibre, Hinke (1970) presents some convincing evidence for at least 25% cellular water being non solvent. Recently, Guidoni et al (1985) using ^{1}H and ^{2}H NMR reported on the changes in dynamic structure and relative amounts of three kinds of water: free, bound and trapped in multilamellar liposomes and concluded that an increase in the amount of bound water appears to be accompanied by an equivalent decrease in the amount of trapped water. Because of the uncertainty of the presence of bound and/or non solvent water, cellular concentrations of ions and water have been quoted in the more unambiguous units of m mol or ml per weight dry cell solid (dcs) and not mol Kg^{-1} (H_2O).

5.3 RESULTS

5.3.1 Effect of pCMBS on water and ion contents of eel red blood cells

Water and ion (Na^+, K^+ and Cl^-) contents were determined under the influence of pCMBS and in combination with Ouabain and furosemide. The reversal of effects brought about by pCMBS by the action of cysteine was also studied. All experiments were carried out at pH 7.4. Water content is expressed as ml gm^{-1} dry cell solid (dcs) and ions content as mM Kg^{-1} dcs. Errors are quoted as $\pm$ S.D.

In Figure (I) the water content was determined in eel red blood cells at different time intervals post-pCMBS treatment at 12°C. The untreated (control) cells showed an average water content of 2.1 to $\pm$ 0.03 ml gm^{-1} dcs during the experimental period of 5 hours. The addition of 1mM pCMBS to the cells showed an initial slow increase of water content through the first 1.5 hr to 2.3 $\pm$ 0.06 ml gm^{-1} dcs, followed by very rapid accumulation of water to 3.5 ml H_2O Kg^{-1} dcs after 4 hours when 8% haemolysis was observed.

Figure II illustrates graphically the Na^+ concentration in eel red blood cells incubated with pCMBS at 12°C. There is a continuous and rapid gain of Na^+ ions compared to the control levels (50 $\pm$ 5 mM Kg^{-1} dcs) to 396 $\pm$ 20 mM Kg^{-1} dcs after 4 hr at which some haemolysis is observed.

The potassium loss from eel erythrocytes incubated with pCMBS at 12°C is shown in figure III. K^+ efflux occurs instantly starting at a control level of 201 $\pm$ 10 mM Kg^{-1} dcs and reaching 36 $\pm$ 3 mM Kg^{-1} dcs after 4 hours.

The chloride content (Cl) of eel red blood cells post pCMBS incubation at 12°C is shown in figure IV. There is an initial but slow increase in the cellular Cl^- content from a control level of 164 $\pm$ 2.7 mM Kg^{-1} dcs to a level of 373 $\pm$ 12 mM Kg^{-1} dcs 4 hr after pCMBS addition.

Fig I to IV

Changes in water and ion contents with time of eel erythrocytes in isotonic saline solution and treated with pCMBS at 12°C.

Fig I **water content**

Fig II **Na^+ content**

Fig III **K^+ content**

Fig IV **Cl^- content**

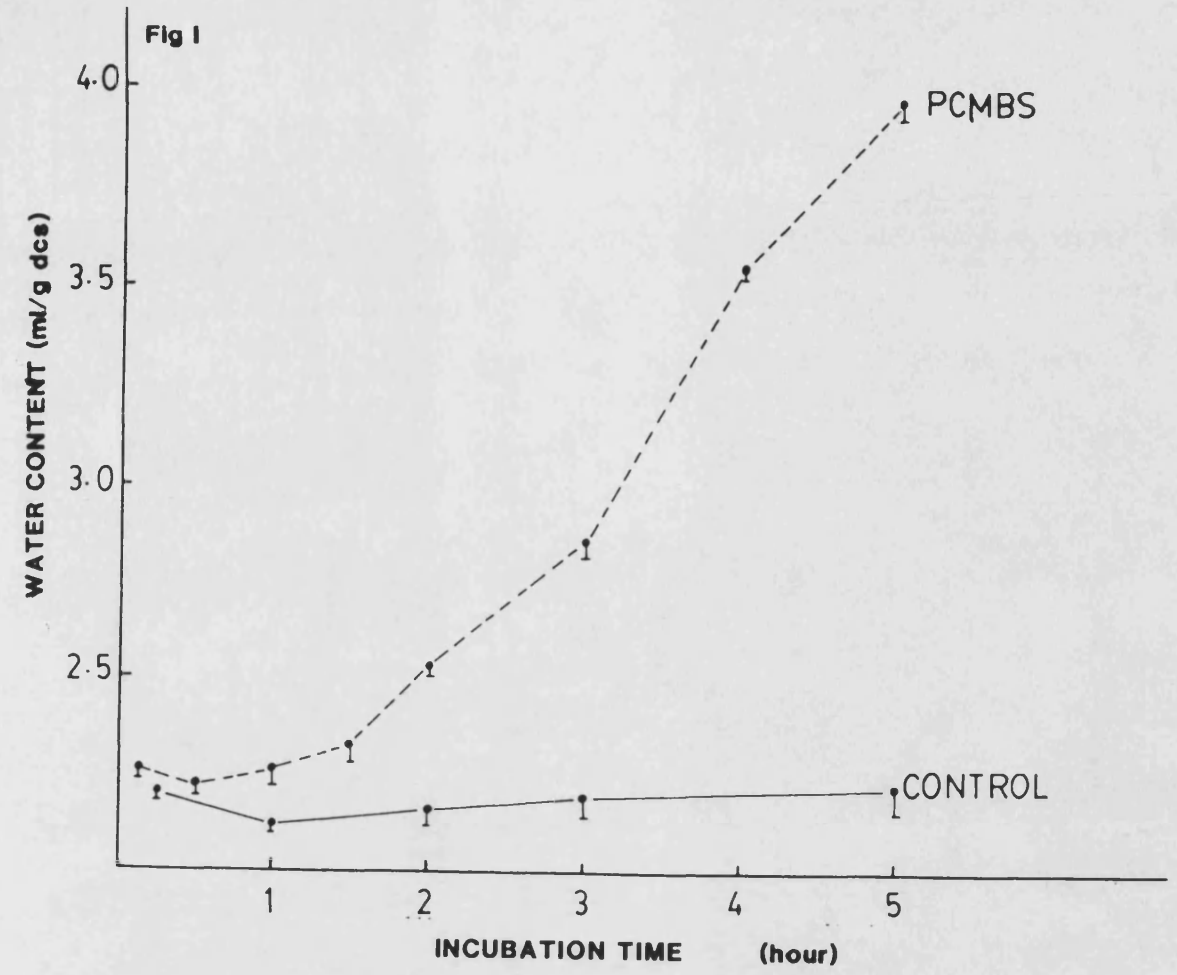


Fig I
4·0
3·5
3·0
2·5
WATER CONTENT (ml/g dcs)
PCMBS
CONTROL
1
2
3
4
5
INCUBATION TIME (hour)

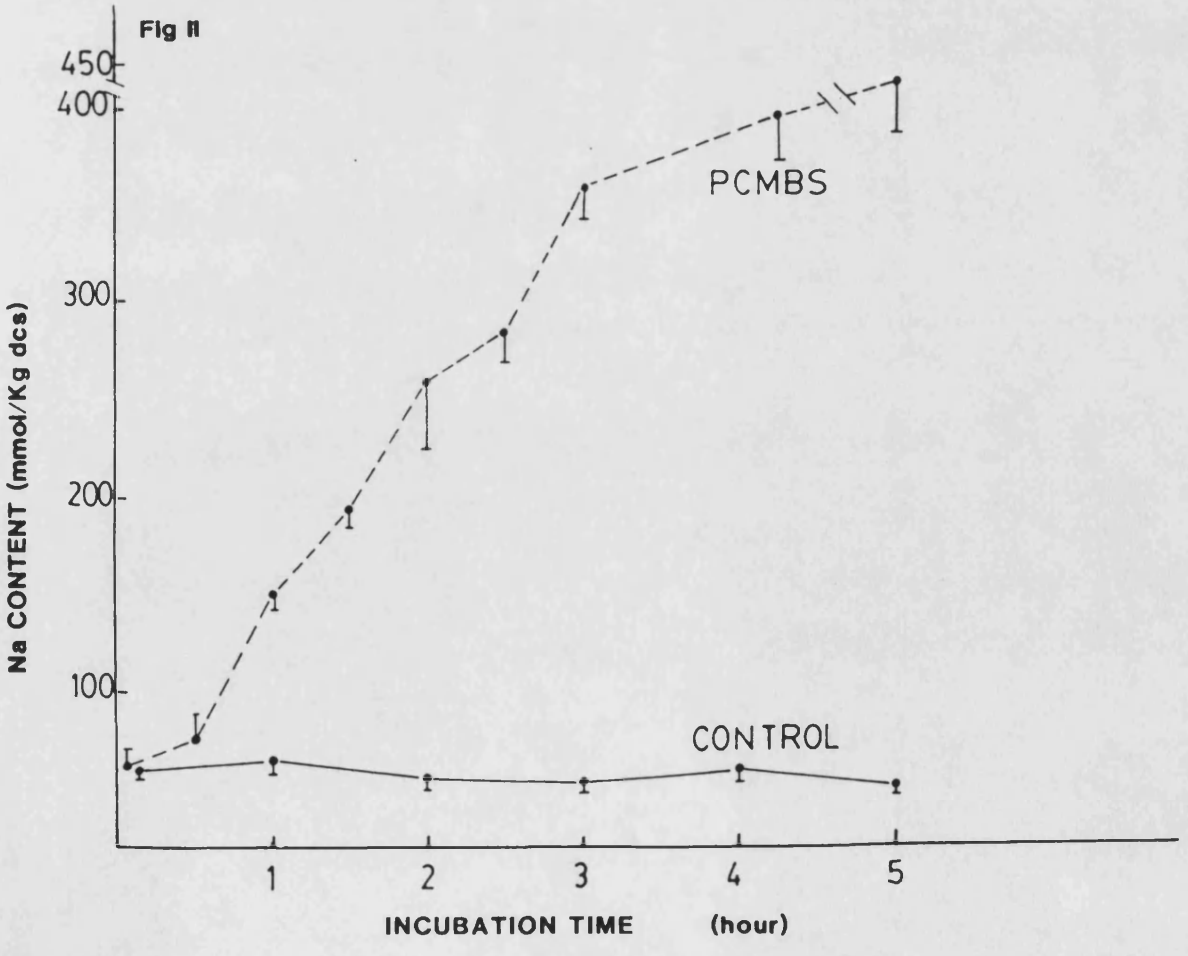


Fig II
450
400
300
200
100
Na CONTENT (mmol/Kg dcs)
PCMBS
CONTROL
1
2
3
4
5
INCUBATION TIME (hour)

Fig III

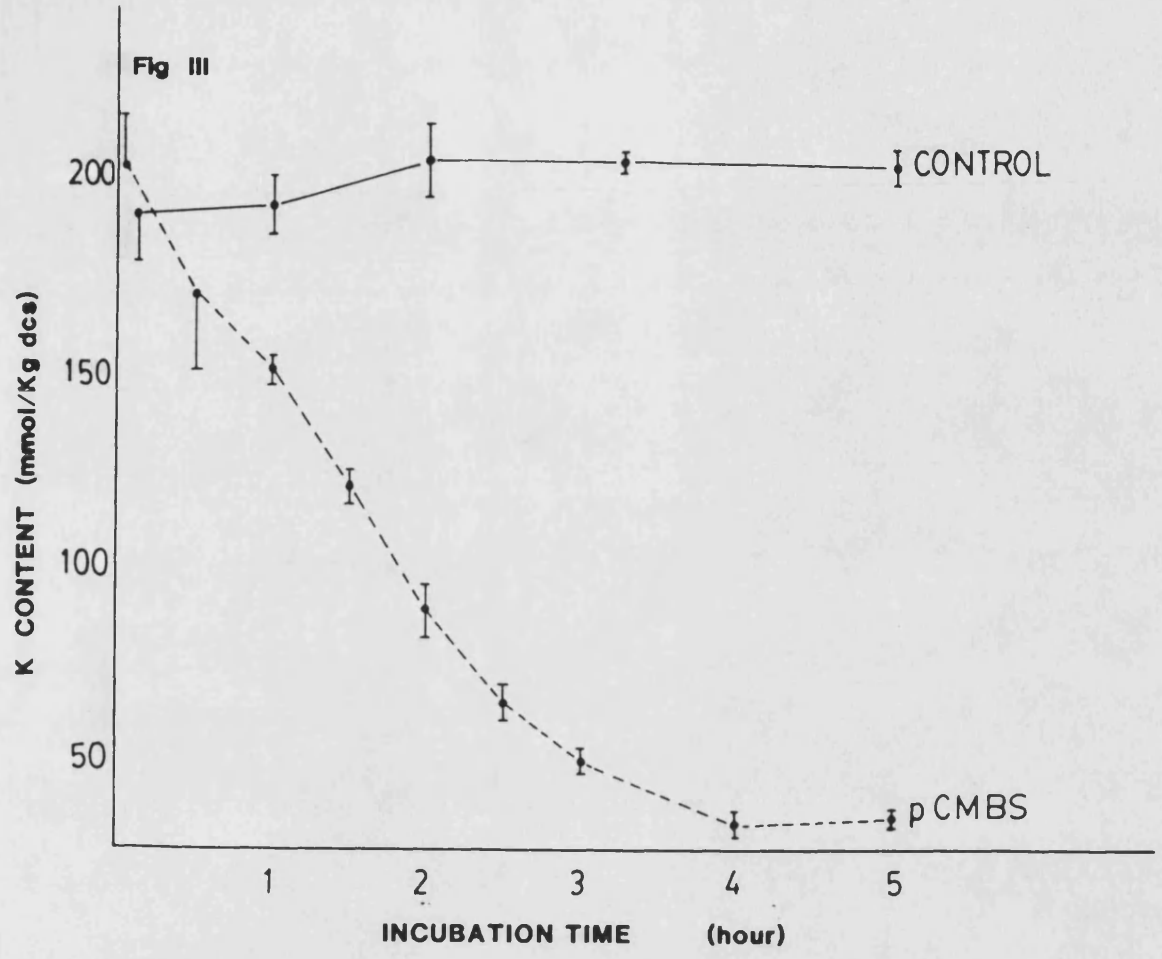
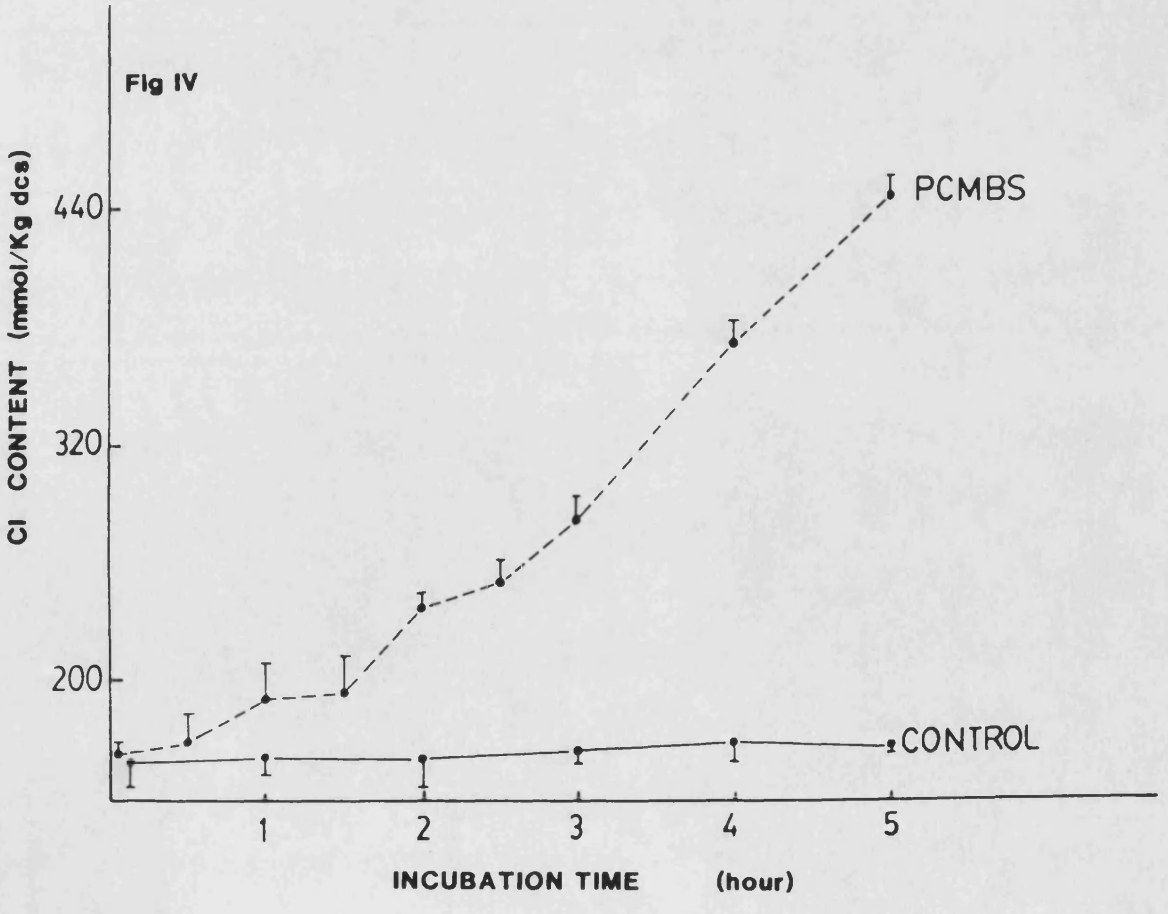


Fig IV



5.3.2 Effect of cysteine on pCMBS action

The development of the reversal of pCMBS induced water, Na^+ and Cl^- gain and K^+ loss by cysteine was investigated by the addition of 10 mM cysteine to pCMBS-treated eel erythrocytes (at 12°C and pH 7.4) at 45 min, 75 min and 2.5 hr after mercurial treatment.

In figure V, the addition of cysteine at both 45 min and 75 min showed a complete reversal of water uptake relative to pCMBS only treated cells followed by a reduced but significant water gain of respectively 0.07 and 0.10 ml gm^{-1} dcs hr^{-1} in the first 2.5 hr after cysteine addition. This may be compared to a water gain of 0.28 ml gm^{-1} dcs hr^{-1} in pCMBS only treated cells. The reversal, with cysteine added 2.5 hr post pCMBS treatment, occurred more slowly than that found with earlier times of cysteine addition and water accumulation is slowed to a rate of 0.19 ml gm^{-1} dcs hr^{-1}.

Slight haemolysis was observed at 20 hrs after cysteine addition in the case of 2.5 hr post pCMBS treated cells compared to complete haemolysis for pCMBS only treated cells at 6 hrs. No haemolysis occurred in the case of early cysteine addition.

The recovery in the case of pCMBS-induced Na^+ influx is shown in figure VI, illustrating that the early addition of

cysteine at 45 min and 75 min slowed down considerably the Na^+ accumulation for 6 hours post pCMBS treatment, followed by complete reversal towards control levels in the following 17 hours. After 2.5 hr of pCMBS treatment the addition of cysteine, although slowing down Na^+ uptake, fails to return it to control levels.

The recovery action of cysteine on the passive K^+ loss is evident and the data is shown in figure VII. The early addition of cysteine (45 min and 75 min) initially stopped K^+ loss for 2 hr after cysteine addition, followed by K^+ gain restoring the K^+ level to within the normal control value (ie complete recovery) after 20 hrs of incubation at 12°C. The recovery with cysteine is more obvious at 45 min than at 75 min post pCMBS. The addition of cysteine at 2.5 hr post pCMBS slowed down the K^+ efflux for 3 hours but was then followed by K^+ gain not reaching control levels.

The pattern of Cl^- recovery after the addition of cysteine to pCMBS treated cells is similar to that found in water gain recovery. The data are presented in figure VIII.

The addition of cysteine 45 min and 75 min to pCMBS treated cells, slowed down the Cl^- uptake more than when cysteine was added at 2.5 hr post mercurial treatment. There was no recovery with the late addition of cysteine.

Generally, it is evident, from figures V to VIII that the reversal by addition of cysteine 45 min or 75 min

post-pCMBS treatment is more effective than the late addition. It is also clear that early desorption of pCMBS by cysteine hampered Na^+ influx and K^+ eflux for 2-3 hours followed by complete recovery, while the influx of water and Cl^- were slowed down without complete reversal.

The recovery in case of Na^+ uptake and K^+ loss could be due to recovery of Na/K active transport.

Fig V to VIII

Effect of Cysteine addition on water and ion contents of pCMBS treated eel erythrocytes at 12 °C.

45 min post mercurial treatment I

75 min post mercurial treatment II

150 min post mercurial treatment III

Fig V water content

Fig VI Na^+ content

Fig VII K^+ content

Fig VIII Cl^- content

Fig V

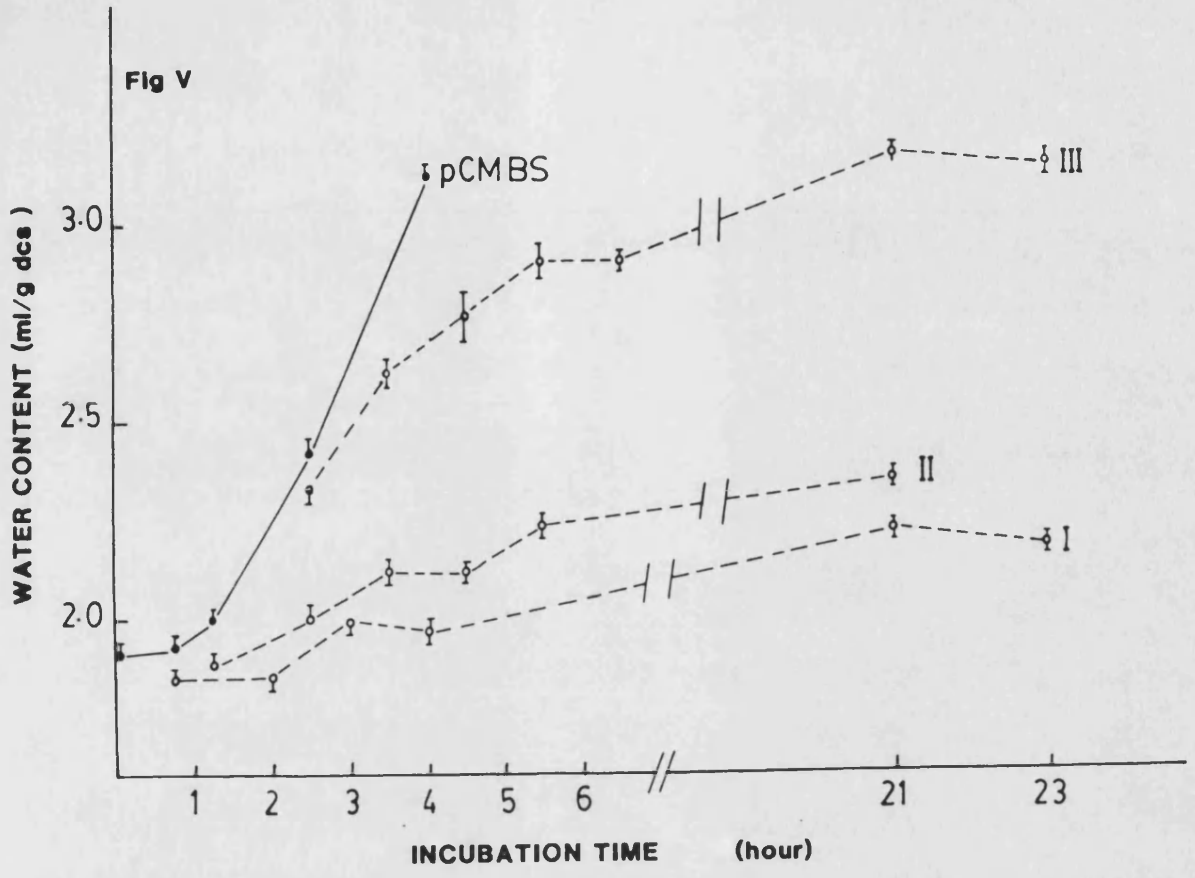
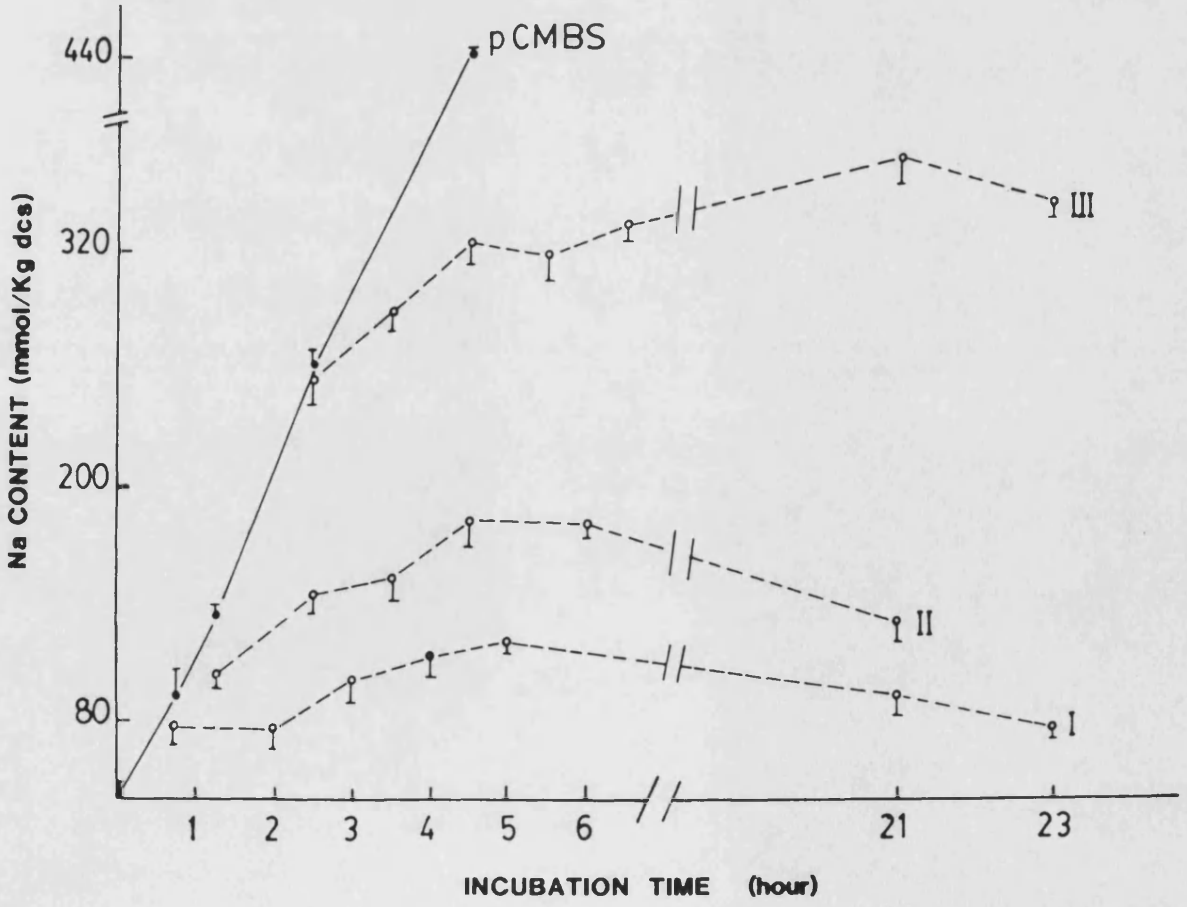
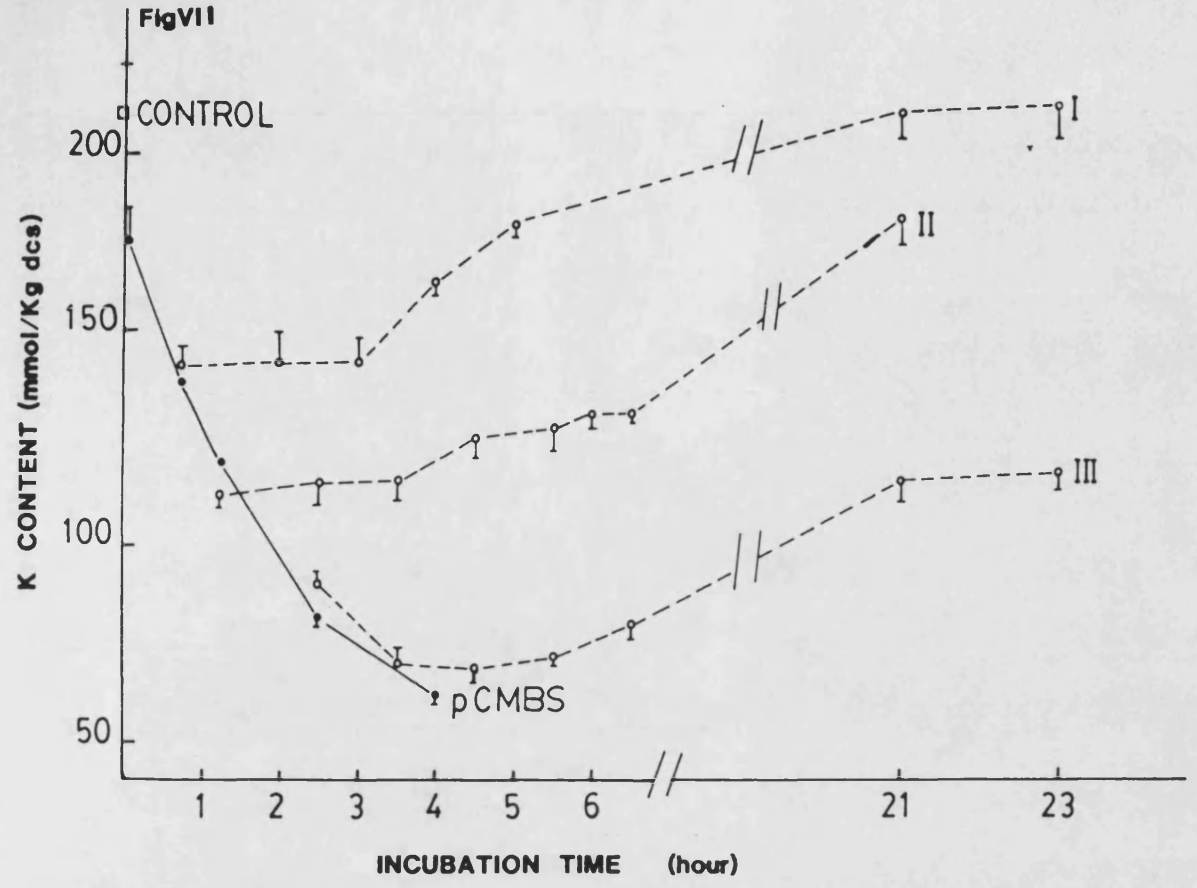

WATER CONTENT (ml/g dcs)
3·0
2·5
2·0
pCMBS
III
II
I
1
2
3
4
5
6
21
23
INCUBATION TIME (hour)

Fig VI


Na CONTENT (mmol/Kg dcs)
440
320
200
80
pCMBS
III
II
I
1
2
3
4
5
6
21
23
INCUBATION TIME (hour)


FigVII
CONTROL
200
150
100
50
K CONTENT (mmol/Kg dcs)
I
II
III
pCMBS
1
2
3
4
5
6
21
23
INCUBATION TIME (hour)

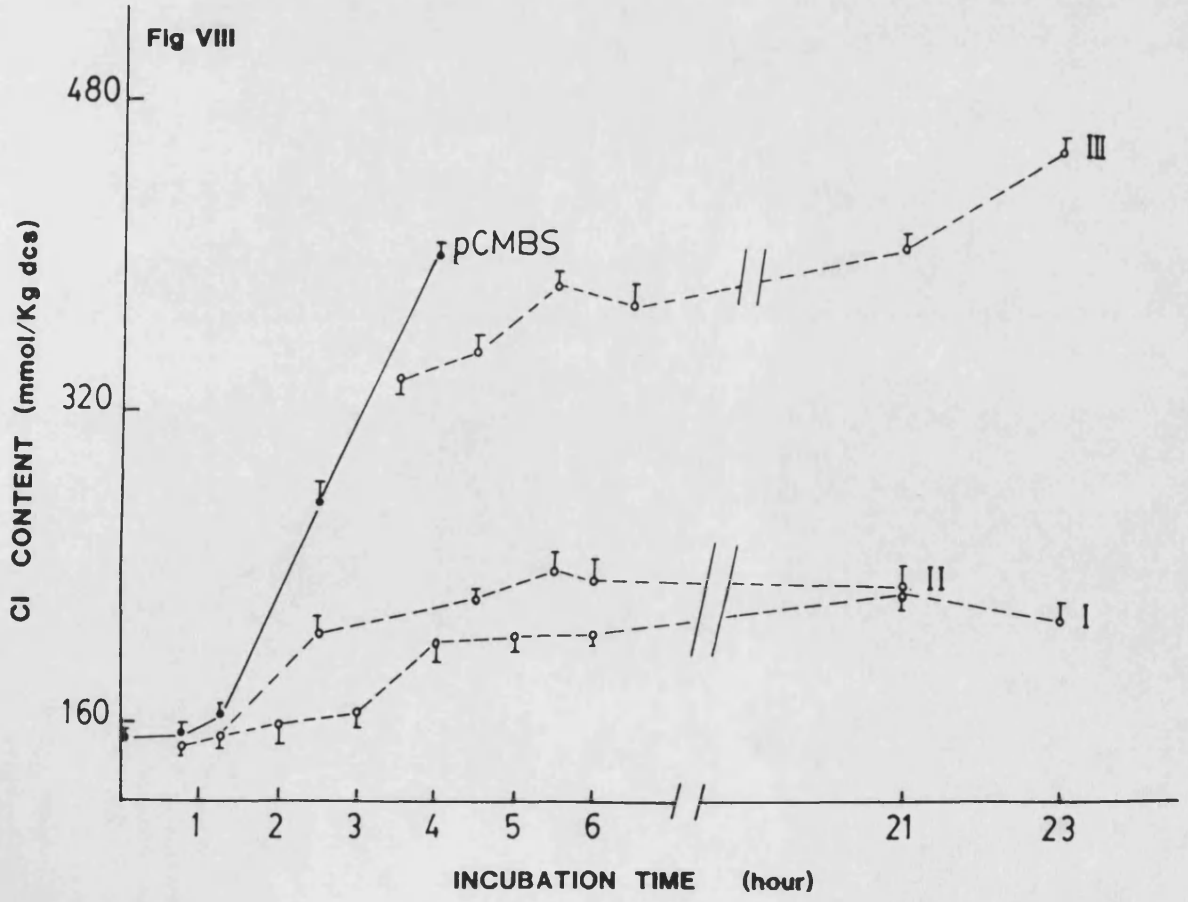


Fig VIII
480
320
160
Cl CONTENT (mmol/Kg dcs)
III
pCMBS
II
I
1
2
3
4
5
6
21
23
INCUBATION TIME (hour)

5.3.3 Effect of pCMBS on ouabain or furosemide treated eel red cells

The action of 1 mM pCMBS on ouabain or furosemide treated eel red blood cells was investigated at 15°C pH 7.4 and the data presented in figures IX to XII. A slightly higher temperature was used to obtain more effective ouabain and furosemide binding.

By inspection of figures (IX to XII) it is evident that the treatment of eel red blood cells with ouabain to stop Na^+-K^+ active transport or with furosemide to inhibit K^+/Cl^- cotransport does not affect the pCMBS action on water and ions movement across eel erythrocyte membrane.

Experimental error bars are not presented so as to eliminate overlapping and confusion of the graphs. All differences with treatment are insignificant.

Fig IX to XII

Effect of pCMBS on water, Na^+, K^+ and Cl^- contents of eel erythrocytes pretreated with 1 mM of ouabain or furosemide.

Fig IX water content

Fig X Na^+ content

Fig XI K^+ content

Fig XII Cl^- content

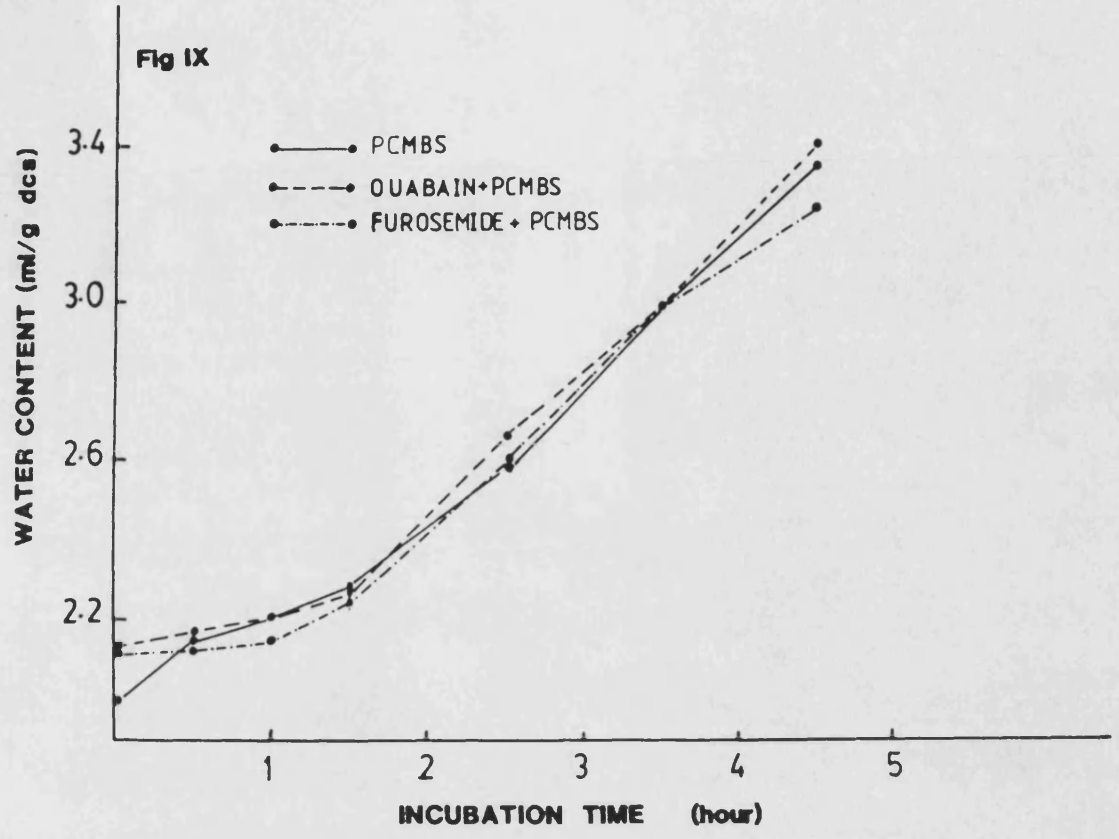


Fig IX
PCMBS
OUABAIN+PCMBS
FUROSEMIDE + PCMBS
WATER CONTENT (ml/g dcs)
3·4
3·0
2·6
2·2
1
2
3
4
5
INCUBATION TIME (hour)

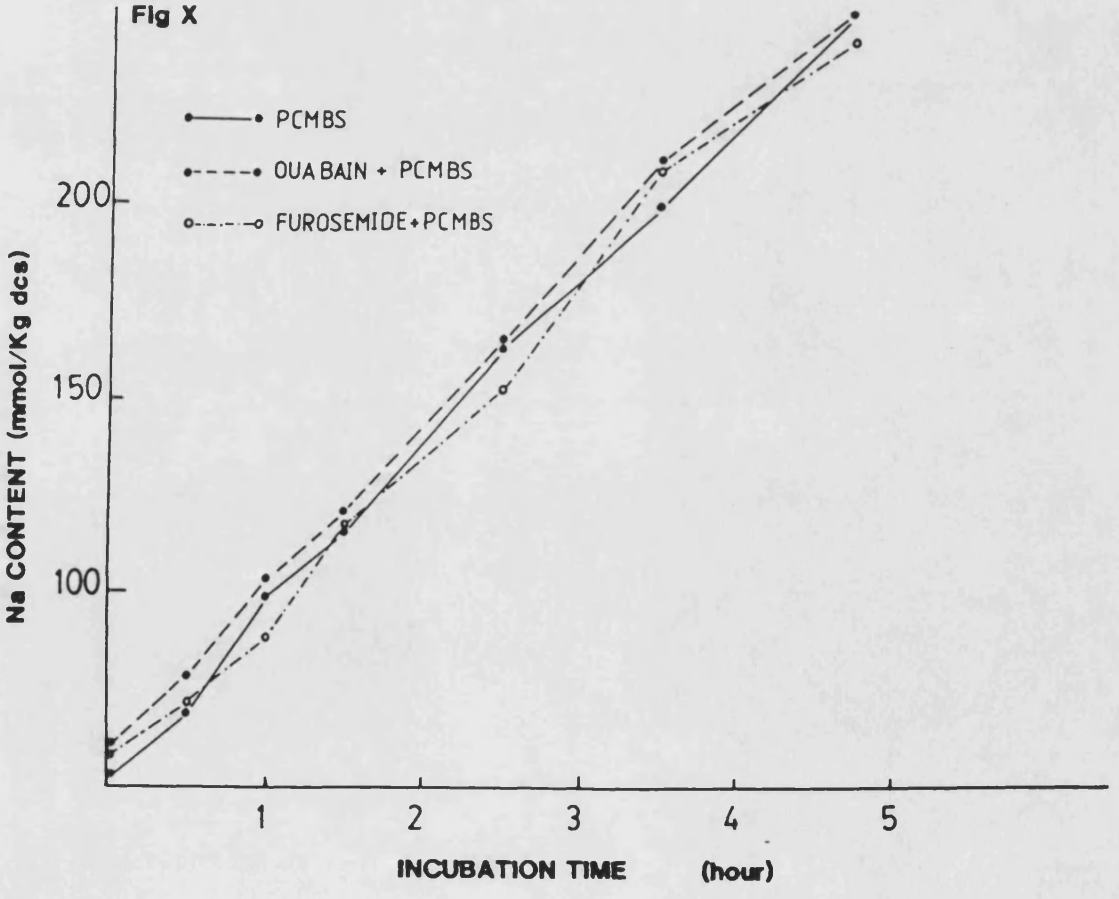


Fig X
PCMBS
OUABAIN + PCMBS
FUROSEMIDE+PCMBS
Na CONTENT (mmol/Kg dcs)
200
150
100
1
2
3
4
5
INCUBATION TIME (hour)

Fig XI

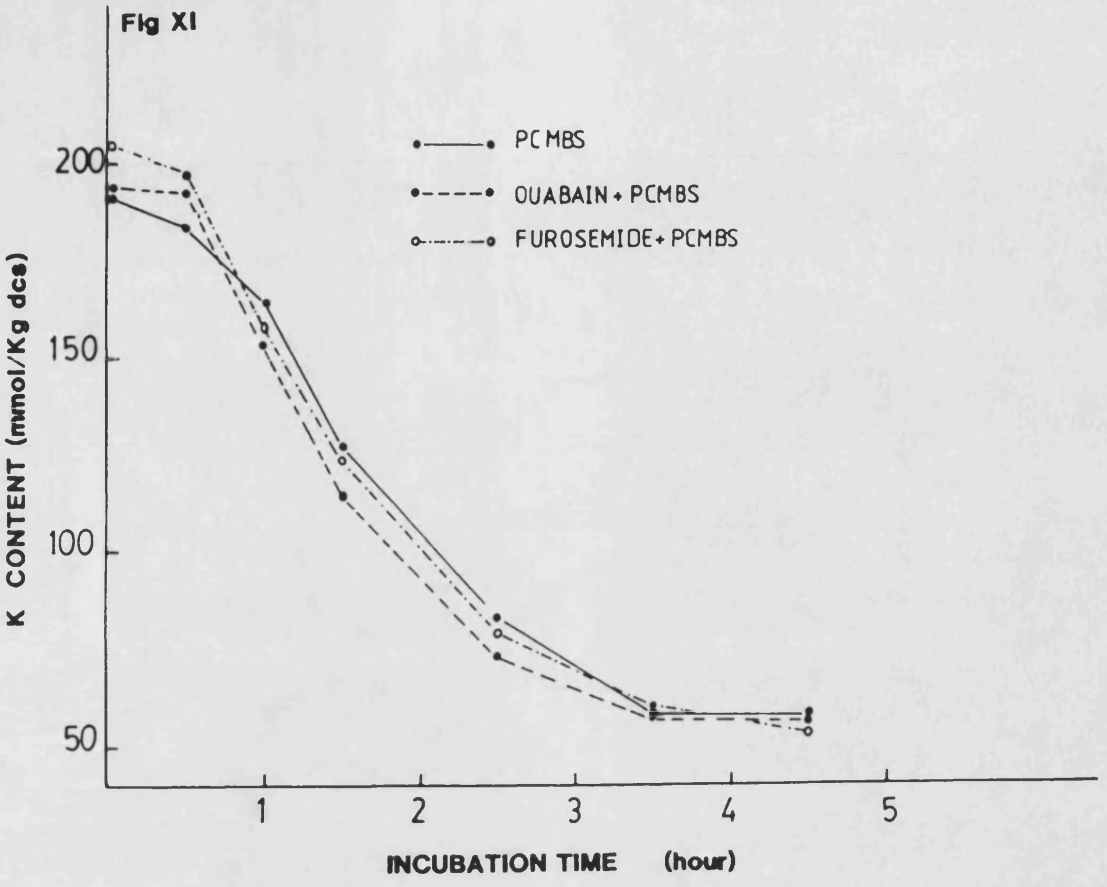
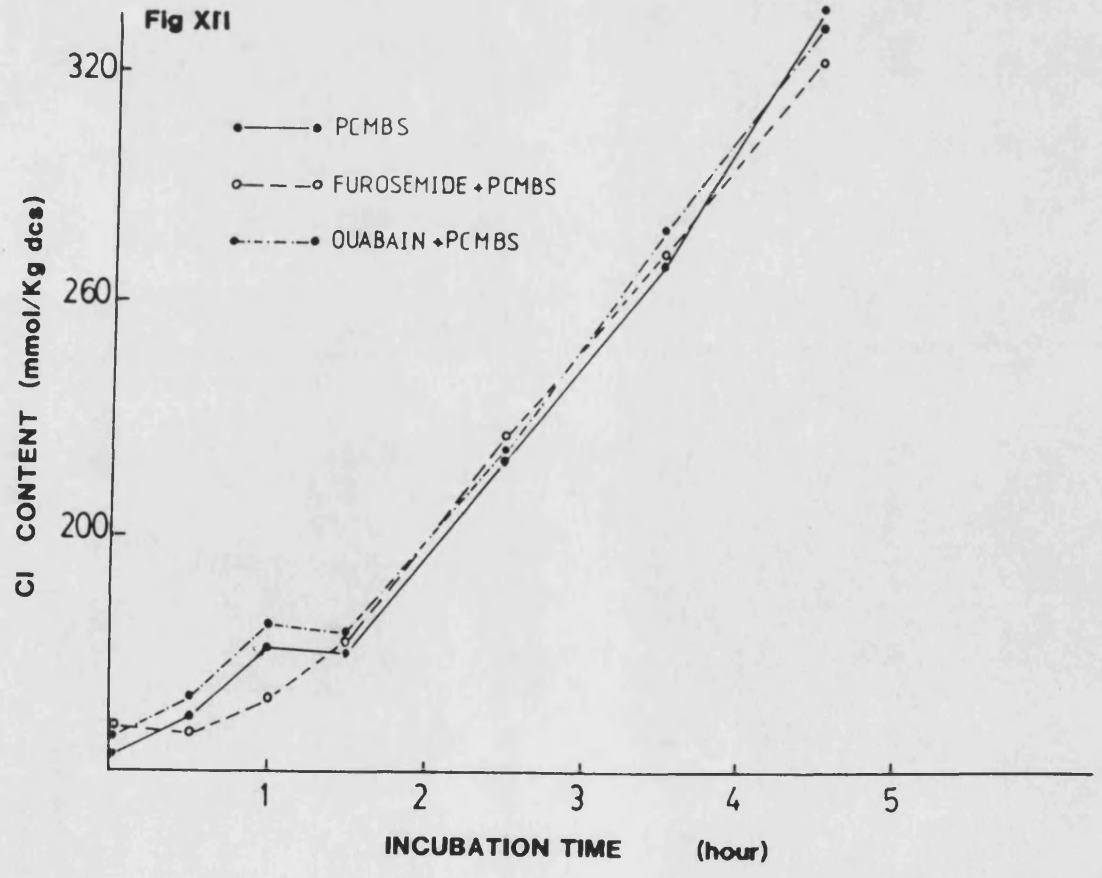


Fig XII



5.3.4 Changes of cell volume of pCMBS treated eel red blood cells

Figure XIII shows the role played by Na^+ ions in cell volume change under the influence of pCMBS at 15°C and pH 7.4. In this experiment, eel red blood cells were suspended in two isotonic solutions of (i) sodium chloride and (ii) choline chloride followed by the addition of 1 mM pCMBS (at pH 7.4) and the change in cell volume assessed using haematocrit as the parameter.

There is a greater increase in the volume of cells suspended in NaCl solution compared to cells suspended in choline chloride solution implying a gain of sodium ions elevating the cellular osmotic content leading to a concomitant water influx and increase in cell volume eventually resulting in gradual haemolysis.

The observed increase in volume of cells suspended in NaCl solution is greater than with cells suspended in choline chloride. The increase in the latter case may be due to the membrane becoming permeable to choline.

The haemolysis of eel erythrocytes was measured at various times during the incubation with 1 mM pCMBS at 15°C and pH 7.4, and the percentage haemolysis (haemoglobin loss) graphically represented in figure XIV.

Haemolysis starts at 3 hr post pCMBS treatment followed by a rapid increase in haemoglobin loss amounting to 85-90% haemolysis after 7 hrs.

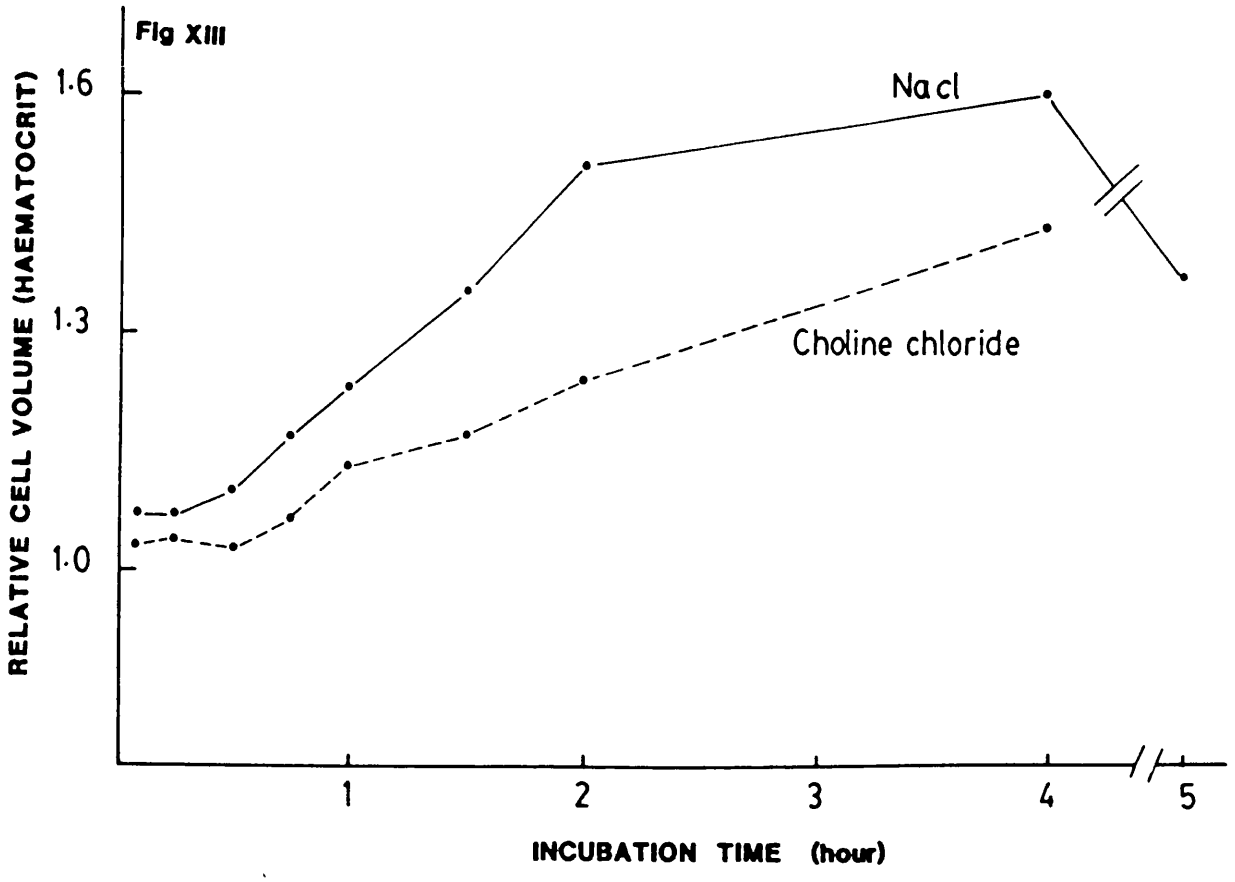


Fig XIII Changes in relative cell volume (Haematocrit) with time of pCMBS treated eel erythrocytes suspended in isotonic NaCl or choline chloride solutions

$$\text{Relative cell volume} = \frac{H_t \text{ of treated cells}}{H_t \text{ of control cells}}$$

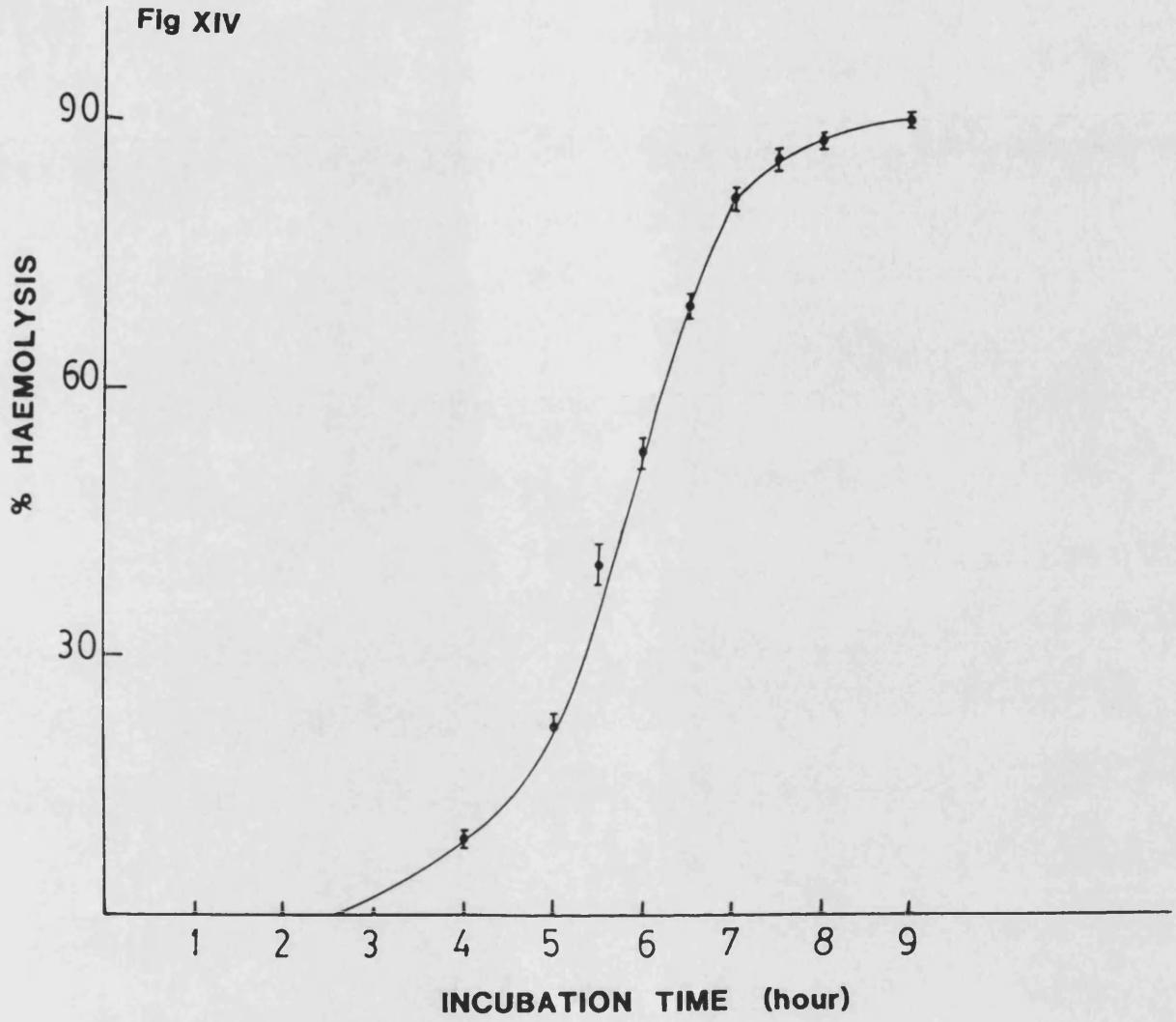


Fig XIV Percentage Haemolysis with time of eel erythrocytes treated with pCMBS.

5.3.5 Effect of pCMBS on water and ion contents of human red blood cells

Treatment of human erythrocytes with the organic mercurial compound pCMBS at 15°C and pH 7.4 showed little change in the water content within the first 6 hours of incubation, as shown in figure XV. After this time there was a gain of water at the 7th hour and continuing for the next 23 hours amounting to a level of 3.3 ± 0.08 ml gm^{-1} dcs compared to control level of 1.92 ± 0.07 ml gm^{-1} dcs. A significant amount of haemolysis was observed by this time beginning 4 hr post pCMBS treatment.

The addition of 10 mM cysteine at 45 min, 90 min and 4 hours post pCMBS treatment inhibited the mercurial induced water uptake and maintained the water level within control limits for at least 30 hours post pCMBS incubation.

The percentage haemolysis starting 4 hrs of pCMBS treatment was held constant by the addition of cysteine.

Figure XVI shows the change in Na^+ levels of human red cells incubated with pCMBS at 15°C and pH 7.4 and the reversal effect of cysteine, when added at different time intervals post pCMBS. It is clear that there is a gain of Na^+ ions at a rate of 6.6 mM Kg^{-1} dcs hr^{-1} for the first 1.5 hrs followed by a more rapid influx of 14.5 mM Kg^{-1} dcs hr^{-1} for the following 7 hours of incubation. After 30

hrs, the Na^+ level reaches 220 mM Kg^{-1} dcs, compared to untreated cells of 40 mM Kg^{-1} dcs, accompanied by considerable cell lysis.

The addition of cysteine at 45 min, 90 min post pCMBS stopped Na^+ gain instantly and maintained constant levels for at least 24 hrs. The later addition of cysteine (4 hrs post pCMBS treatment) reduced Na^+ gain compared to pCMBS only treated cells and maintained constant levels for 1 hr followed by slow increase for the rest of the incubation time.

In comparison with eel cells in which Na^+ gain is immediate and continuous through the whole incubation period, human cells show a lag of 1.5 hrs period before Na^+ gain.

Figure XVII shows the leakage of K^+ from human cells induced by pCMBS at 15°C and pH 7.4. For the first 1.5 hr the K^+ loss was 16 mM Kg^{-1} dcs hr^{-1} followed by increased leakage within the next 5 hour amounting to 25 mM Kg^{-1} dcs hr^{-1}. After 30 hours under the same conditions of incubation there is a small difference (45 mM Kg^{-1} dcs) compared to the observed level at 7 hr post pCMBS treatment (53 mM Kg^{-1} dcs).

The presence of cysteine after 45, 90 min and 4 hrs of pCMBS addition, stopped K^+ loss immediately followed by an insignificant change in cellular K^+. In all cases, unlike the eel cells, after 24 hours incubation, the K^+ levels

failed to return to control levels.

The Cl^- content of human red cells under the influence of pCMBS at 15°C is illustrated in figure XVIII and shows a constant level for Cl^- ions for 3.5 hours post incubation, followed by rapid influx which continues until 30 hours post treatment when considerable haemolysis was observed.

The addition of cysteine at either 45, 90 min or 4 hr post pCMBS treatment does not affect the rate of Cl^- gain for 7 hrs, only showing a reversal in Cl^- uptake by 24 hrs. The final Cl^- level achieved is still higher than that of the control. The reversal effect on Cl^- gain in the case of human red cell is very slow compared to that in eel but when it does occur it is more effective.

Figs XV to XVIII

Effect of pCMBS on water, Na^+, K^+ and Cl^- contents of Human erythrocytes and reversal by cysteine added at different time intervals, 45 min (I), 90 min (II) and 4 hours (III) post mercurial treatment, at 15°C.

Fig XV **water content**

Fig XVI **Na^+ content**

Fig XVII **K^+ content**

Fig XVIII **Cl^- content**

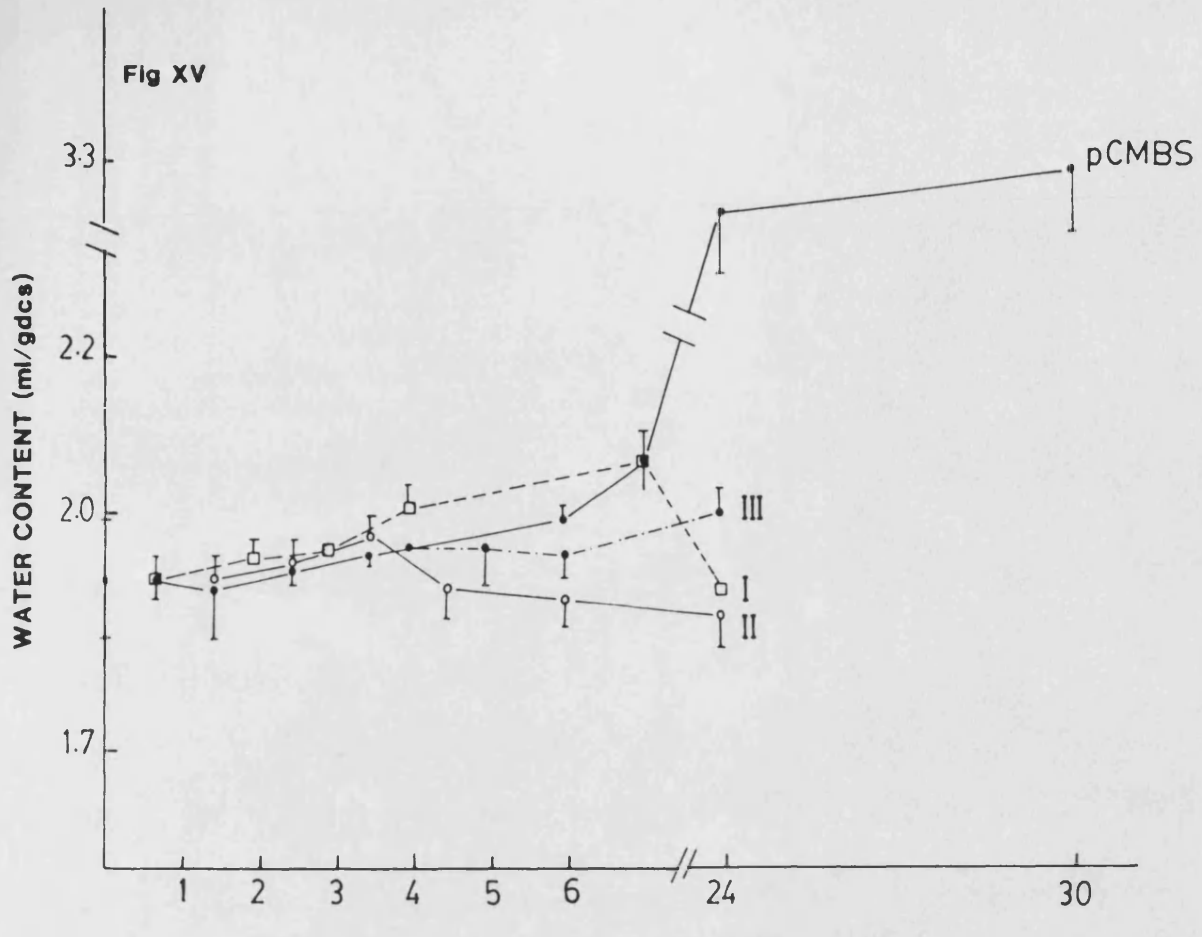


Fig XV
WATER CONTENT (ml/gdcs)
3·3
2·2
2·0
1·7
pCMBS
III
I
II
1
2
3
4
5
6
24
30
INCUBATION TIME (hour)

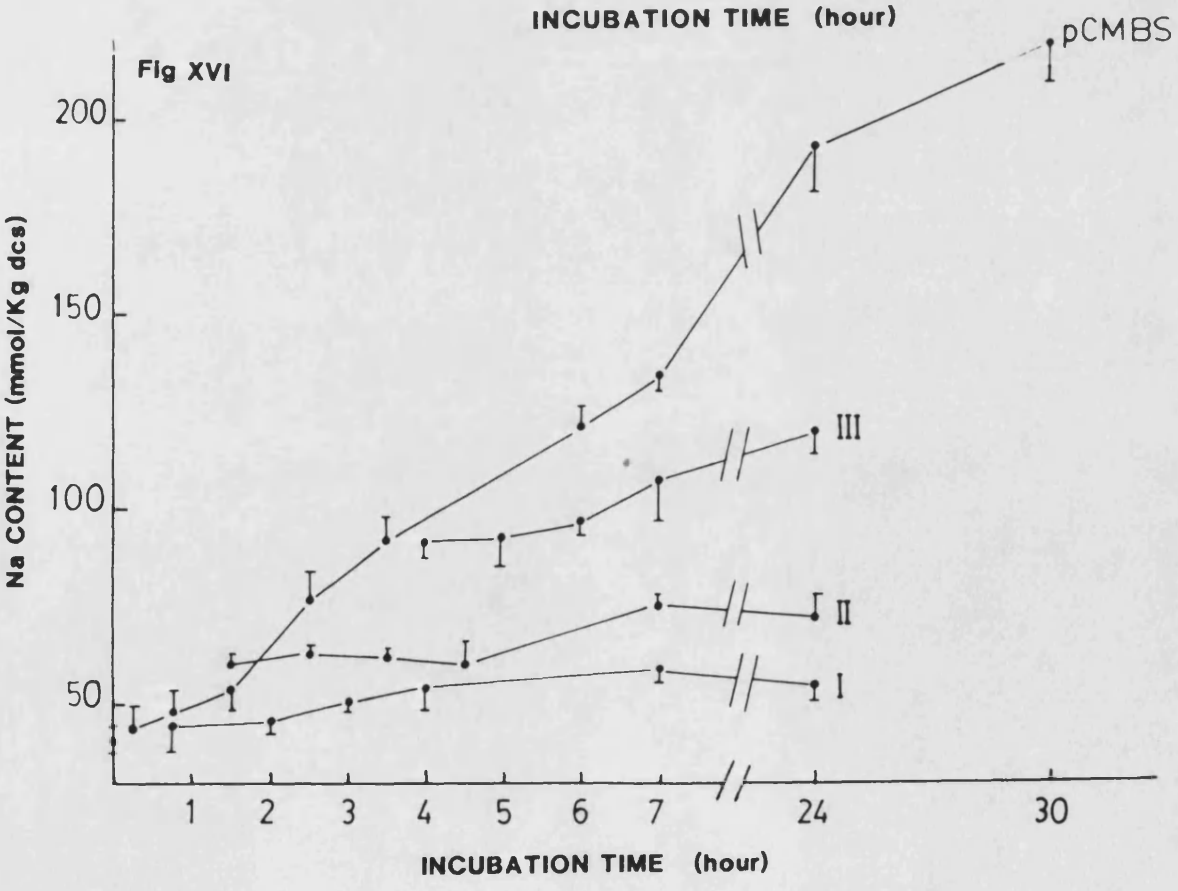


Fig XVI
Na CONTENT (mmol/Kg dcs)
200
150
100
50
pCMBS
III
II
I
1
2
3
4
5
6
7
24
30
INCUBATION TIME (hour)

Fig XVII

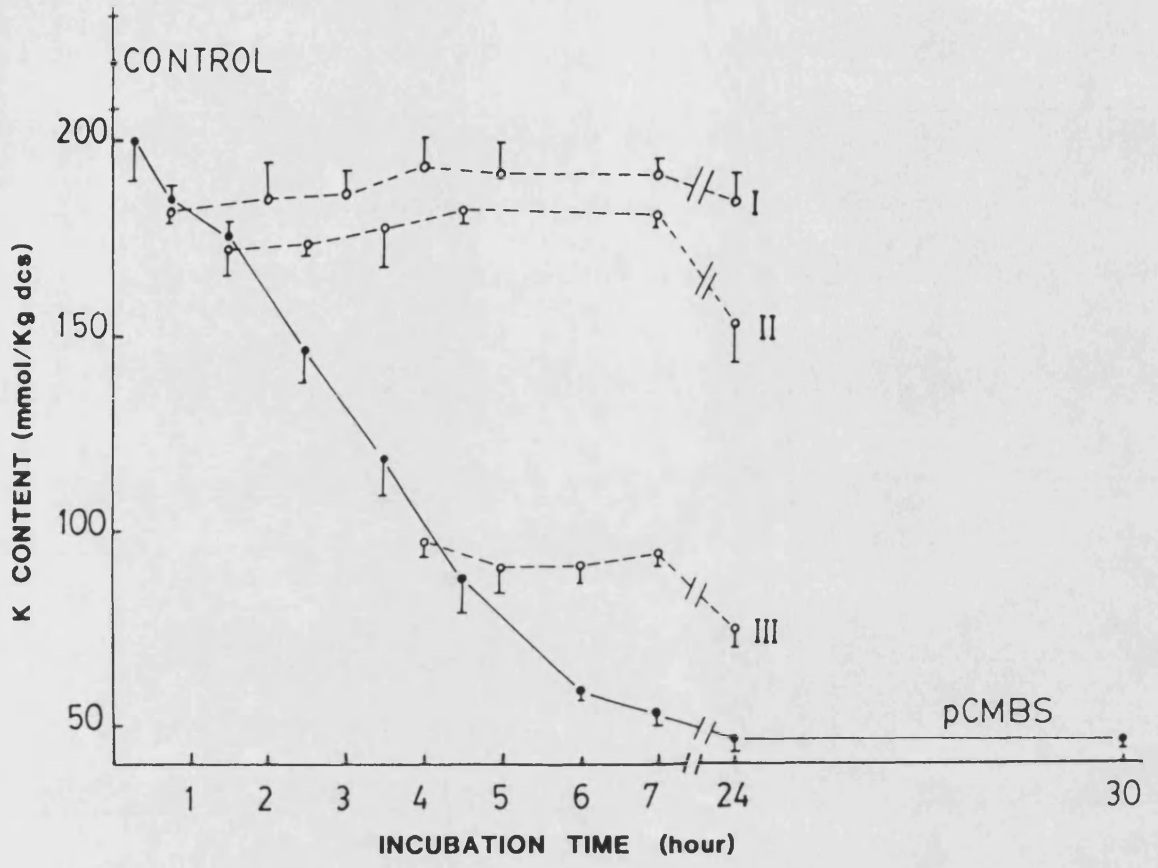
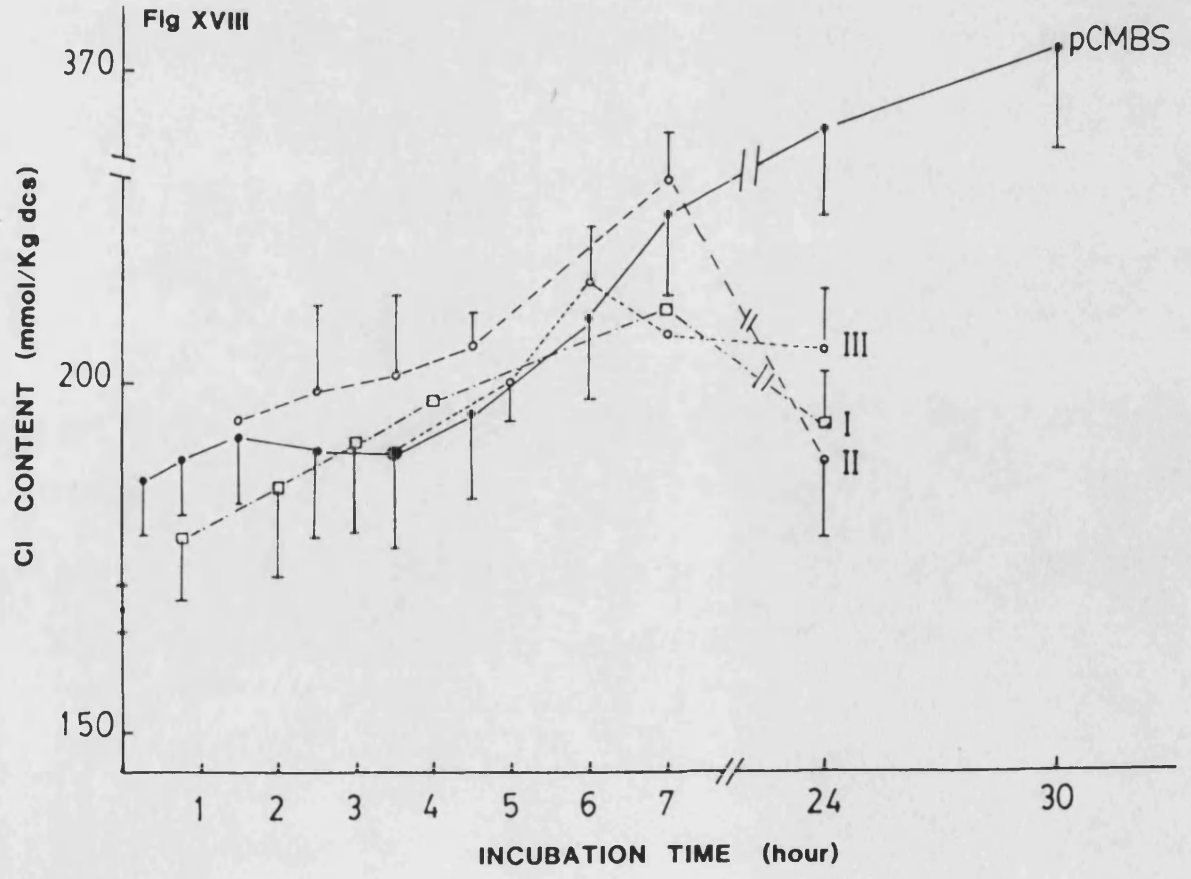


Fig XVIII



5.4 DISCUSSION

The specific sulphydryl organic mercurial, pCMBS, has a similar effect on the movement of both water and ions across theerythrocyte membranes of eel and human. In both cells at 15°C it increases the rate of uptake of water, Na^+ and Cl^- and the loss of K^+, eventually resulting in haemolysis. The effect is in accord with the results produced by similar agents (Sutherland et al (1967), Rega et al (1967) and Shapiro et al (1970)) on cation movement in human erythrocytes at 2, 25 and 37°C.

The rate of water influx through pCMBS treated eel red cell membranes can be divided into two phases; an initial slow uptake for the first 1.5 hr post addition of the slowly penetrating mercurial pCMBS and a second phase of rapid water gain over the following 3-4 hours, (2 to 6 fold at 15°C and 12°C respectively). This would be consistent with the finding (described in chaps 3 & 4)of two populations of membrane -SH groups differing in their location within the eel red cell membrane.

In the case of human cells, there is no change in cellular water content for the first 6 hrs post pCMBS treatment. There is then a rapid influx of water followed by haemolysis which is progressive over the following 24 hrs.

These human data are in agreement with the slow increase observed in the volume of human erythrocytes suspended in

isotonic NaCl solution (Sutherland et al (1967) and can be compared with the rapid increase (observed in this study) in the volume of eel red blood cells suspended in solutions of similar concentrations. Also these data are in accord with the observation of 80% haemolysis of eel erythrocytes within 7 hr compared to only 10% in human erythrocytes 10 hr post pCMBS treatment (Sutherland et al, 1967).

The delayed effect of pCMBS on Na^+ gain, for human cells relative to eel may be attributed to and explained by a slow penetration of pCMBS through the human red cell membrane (Shapiro et al, 1970) compared to a relatively faster permeability across the eel erythrocyte membrane. This suggestion would also give some insight as to the depth in the membrane of the critical -SH groups. They are perhaps deeper in the case of human than in the eel red blood cell membrane. This possibility is supported by the finding (Chapter 3) that the critical -SH groups for water control are not in direct contact with the external medium and is in agreement with the findings of Grinstein and Rothstein (1978).

There is a dearth of literature data available for the effect of pCMBS on Cl^- movement. In this study the cellular chloride content increased when erythrocytes from human and eel were incubated with pCMBS. The pattern of Cl^- uptake is similar to that for water and is related to the electrochemical gradients generated. This may be explained by the following analysis in which the ion

concentrations are converted to units of mMl^{-1} so that the gradients relative to the suspending medium are more meaningful.

5.4.1 Analysis of ion and water movement in cells treated with pCMBS and Cysteine

After 45 min of pCMBS treament (fig V to VIII and Scheme A) there is an equal loss and gain of K^+ and Na^+ (1:1) across the eel erythrocyte membrane (Scheme A) with an insignificant Cl^- movement. Because of this simple 1:1 exchange of cations, the water volume remains constant.

The Na^+/K^+ exchange does not appear to be linked because in both eel or human cells, their rates of movement are dissimilar over the first 15 minutes of preparation, K^+ being lost immediately and Na^+ after delay.

In human erythrocytes (fig XV to XVIII Scheme B) 45 minutes post-pCMBS treatment, there are minor changes in the ion and water levels and it is only after 6 hrs that there has been a significant cationic (but little anionic) disturbance with a greater K^+ loss than Na^+ gain (2 K : 1 Na. On a simple ionic addition basis to reflect approximately the osmotic potential within the cell, there is little change and hence water volume change. The ionic movements that occur in human cells probably reflects the simple diffusional rates of the ions ($P_K > P_{Na}$).

After 2.5 hours incubation of eel red cells in pCMBS, the Na^+ gain increases over K^+ loss and is accompanied by Cl^- gain. This brings about an elevated intracellular ion concentration causing an osmotic flow into the cell.

In human cells the ionic system appears to be simply running down bringing little change in total intracellular ionic concentration and therefore little osmotic flow of water into the cell.

This is in direct contrast to the eel cells in which, after approximately 3 hrs of pCMBS treatment there is:

a loss of 50 m mol K^+ Kg^{-1} dcs
a gain of 150 m mol Na^+ Kg^{-1} dcs
a gain of 110 m mol Cl^{-1} Kg^{-1} dcs and
a gain of 450 m mol H_2O Kg^{-1} dcs

In total approximately 100 mM of cation/anion is gained coupled with 450 ml H_2O, i.e; 220 m mol Kg^{-1} (H_2O), a value in excess of isotonic water flow. Paradoxically then, Na^+ going inwards, down its concentration gradient, moves faster than K^+ moving outwards down its gradient. If pCMBS opens ion channels, then, like human cells, it would be expected that ion movement will follow physical diffusion laws with K^+ ions moving faster than Na^+. As this is not the case, we must speculate that there could be a change in charge on the haemoglobin molecule making it perhaps more -ve and hence drawing Na^+ in down both an electrochemical and

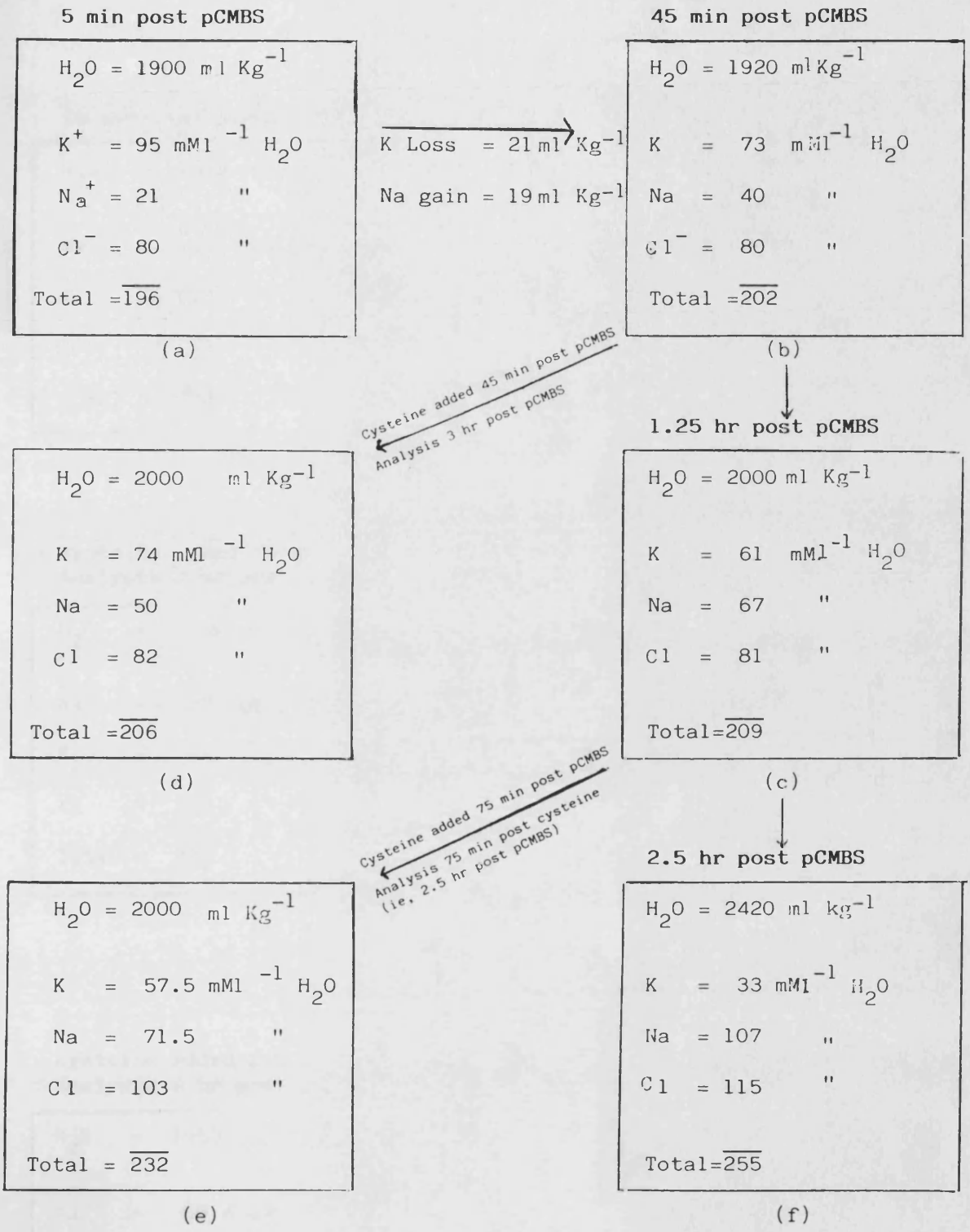
concentration gradient. It is however difficult to reconcile this with why Cl^- should move so effectively in against such an opposing charge even though it would move down its concentration gradient.

There is always the possibility that such rapid and dramatic membrane changes in the eel cells may result in a Na/K pump reversal causing the extensive water movement and lysis so typical of these otherwise fairly resilient cells. The pump is particularly active in these cells relying on aerobic processes (see Chapter 6) and any pump reversal, a not unknown phenomena (Glynn et al (1970) , Glynn and Lew (1970) and Lant et al (1970) would result in a rapid cell swelling and lysis. It would be valuable to explore this suggestion further.

With the addition of cysteine to eel erythrocytes (Scheme A, d & e), the loss or gain of ions are slowed parallel with a slowing down of water movement. The same effect with cysteine was observed with human cells, (d & e in scheme B). In eel erythrocytes, however, intracellular ions always remain elevated, compared to the value at time zero and therefore water continues to enter the cell until lysis occurs. The treatment of such cells with cysteine 2.5 hrs hrs post pCMBS fails to bring about recovery and lysis occurs. This is not so with cells treated up to 1.25 hrs post pCMBS, (e in scheme A) for recovery is effective and haemolysis does not occur.

Eel data from Fig V to VIII

Scheme (A)



*TOTAL is to reflect osmotic potential and is the sum of major cations and anions

Human data from Figs XV to XVIII

Scheme (B)

10 min post pCMBS

H_2O	=	1920 ml kg^{-1}	
Na	=	22	mM l^{-1}
K	=	104	"
Cl	=	97	"
Total	=	223	

(a)

45 min post pCMBS

H_2O	=	1920 ml Kg^{-1}	
Na	=	25	mM l^{-1}
K	=	95	"
Cl	=	99	"
		219	

(b)

+ cysteine added 45 min post pCMBS
Analysis 7 hr post pCMBS

H_2O	=	2050 ml Kg^{-1}	
Na	=	29	mM l^{-1}
K	=	93	"
Cl	=	101	"
Total	=	223	

(d)

6 hrs post pCMBS

H_2O	=	2000 ml Kg^{-1}	
Na	=	60	mM l^{-1}
K	=	30	"
Cl	=	102	"
Total		192	

(c)

cysteine added 2.5 hr post pCMBS
Analysis 6 hr post pCMBS

H_2O	=	1950 ml Kg^{-1}	
Na	=	56.4	mM l^{-1}
K	=	49	"
Cl	=	105	"
Total	=	210	

(e)

Addition of cysteine to human cells 4 hrs post pCMBS treatment fails to induce recovery while its addition up to 2.5 hrs post pCMBS can restore reovery with no haemolysis.

The development and reversal of pCMBS action allows further conclusions to be made concerning the location of sites responsible for the permeability functions of the membrane. Because, in this study, the ion and water permeability of erythrocyte membranes is rapidly reversed by the highly permeant cysteine, but only slowly reversed by non-penetrating albumin (Grinstein and Rothstein, 1978), it may be suggested that the pCMBS-reacting sites, involved in ion permeability, are located within the membrane separated from both the inside and outside environment by a permeability barrier to pCMBS.

Under the influence of pCMBS, the ouabain insensitive and furosemide-insensitive passive permeabilities were increased at the same rate as pCMBS treated eel red cells only. This is in agreement with the findings of Rega et al (1967) who showed that K^+ loss in human red cells is reversible by cysteine addition. One can suggest that pCMBS is working in a similar way to ouabain and furosemide but differs in its reversibility by cysteine.

The mechanism by which pCMBS acts is still uncertain. It does not appear to depend on any inhibition of metabolic activities because red cell glycolysis was found not to be affected by pCMB (Jacob and Jandl, 1962) which penetrates

more rapidly than pCMBS but has the same reactivity with membrane sulphydryl groups, (Van Stevenink et al, 1965).

The passive or downhill movement of cations produced by pCMBS treatment could be attributed to a combination of factors involving the inhibition of ATPase (Skou, 1963) necessary for active transport and to conformational or distortional change produced by membrane SH cross-linking which leads to opening of the ion channels and even a reversal of the pump in eel cells.

It is obvious that the membrane structure with particular regard to their -SH groups is significantly different in eel cells because pCMBS treatment leads to rapid ion and water changes whereas in human such changes bringing about haemolysis occur after delay of some 7-10 hours.

Chapter 6

Effect of ionizing radiation on the Membrane -SH groups of eel and human red blood cells

6.1 INTRODUCTION

The increasing presence of ionizing radiation associated with commercial and medical use necessitates a clear understanding of its damaging effects. Although its actions on the cell nucleus and cytogenetic processes have been widely investigated, its action on the cell membrane and cellular ionic equilibria has received much less attention. Because of some of the documented changes in proteins and lipids exposed to ionizing radiation, an investigation of irradiated cell equilibria may be useful in understanding the mechanisms responsible for the maintenance of membrane integrity.

6.1.1 Radiation Damage to Membrane Components

There is a growing literature on the wide variety of effects that have been observed on irradiation of isolated membranes and whole cell systems, including peroxidation of unsaturated lipids (Myers and Bide, 1966), increased permeability of membranes to ions (Sutherland et al, 1967) and changes in electrophoretic mobility and inactivation of membrane bound enzymes (Edwards et al, 1984). Of importance to this investigation is the report that protein sulphydryl groups are oxidized by irradiation (Shapiro and Kollmann (1968) and Sutherland and Pihl (1968)) and that this oxidation plays a large part in the radiation damage observed in cells. Conversely the presence of compounds having reduced sulphydryl groups appear to be radioprotective.

The mechanism proposed by Howards-Flanders (1960) to explain radioprotectivity was that the sulphydryl groups could donate a hydrogen atom to a DNA molecule which had lost one as a result of radiation damage, thus restoring the status quo. Hutchinson (1961) found that the relative transforming ability of Pneumococcus DNA was partially protected from radiation damage by glutathione, particularly in an atmosphere of nitrogen, but in the absence of glutathione the DNA was equally radio-sensitive in either nitrogen or oxygen.

Myers and Bide (1966) showed that irradiated erythrocytes lost intracellular potassium and haemoglobin during post irradiation incubation for 20 hrs and lipid peroxides occurred both immediately after irradiation and during post irradiation incubation at either 4°C or 37°C. Since membranes are rich in lipids they considered that the permeability damage observed after irradiation might result from such lipid oxidation. The earlier work of Robinson (1966) has drawn attention to a possible relationship between lipid peroxidation and damage to sulphydryl groups and this was developed by Sutherland and Pihl (1968) in their experiments to correlate -SH loss and lipid peroxidation in irradiated intact human cells and isolated ghosts. They found that with intact cells little or no peroxidation of membrane lipids could be detected after doses of less than 1000 Gy, however with higher doses, appreciable peroxidation occurred but only when the most radiosensitive sulphydryl groups had disappeared. Earlier

confirmation of this work was by Myers and Slade (1967) who found that the peroxidation of erythrocyte lipids during post irradiation incubation was accelerated in erythrocytes pretreated with the sulphydryl group blocker, iodoacetamide.

When erythrocyte ghosts were irradiated, considerable peroxidation occurred even after moderate radiation doses (below 400 Gy) and this was attributed by Sutherland and Pihl (1968) to a structural modification which occurred in the membranes during preparation rendering them more susceptible to peroxidation. In contrast to the conclusion of Myers and Bide (1966) it appears that peroxidation of membrane lipids does not account for radiation induced permeability changes as these latter occur after doses too small to cause lipid peroxidation.

It has been shown by Archer and Wills (1973) that irradiation or N-ethylmaleimide treatment of HeLa cells is accompanied by a loss of about 50% of the membrane sulphydryl groups and they suggested that there are two distinct populations of membrane sulphydryl groups differing in their radiosensitivity. Sutherland and Pihl (1968) earlier confirmed that the radiation induced loss of sulphydryl groups is mainly due to disulphide formation and that loss of SH groups increases with dose (Sutherland et al, 1967). The corresponding increase in disulphide formation by direct measurement using DTNB suggested that at least 40 per cent of the decrease in erythrocyte

membrane -SH groups is accounted for by disulphide formation and that the loss was even more marked in protein extracted from ghosts than ghosts or intact erythrocytes. This difference can be attributed to the protective effect of intracellular protein, primarily haemoglobin. The disparity between the extent of sulphydryl group destruction in irradiated ghosts and membrane protein may also be attributed to protection by lipids either by direct absorption of radiation energy and/or by covering of the -SH groups in the lipoprotein complex so that they are physically inaccessible to the damaging products (free radicals) of irradiation.

Radiation induced loss of membrane bound sulphydryl groups in Saccharomyces cerevisiae cells was observed by Rink (1975) who showed that only 30-40% of total detectable sulphydryl content was oxidized by radiation even with doses up to 5000 Gy . The loss of sulphydryl groups increases with doses up to 4000 Gy, but at higher doses there is an equilibrium between the oxidation of sulphydryl groups and radiolysis of disulphide groups and thus no further loss of sulphydryl groups can be observed.

The radiation damage to the sulphydryl groups of human erythrocyte membranes that has been extensively studied by Sutherland and Pihl (1968) in whole cells or isolated ghosts and has provided numerical data for D_{37} (ie dose to damage 37% of structure) analysis. For example from the two phase dose-response curves (typical of two populations

of -SH groups), values for D_{37} of 380 Gy and 13700 Gy were derived for intact cells and 180 Gy and 2800 Gy for ghosts. Interestingly freezing and thawing of ghosts prior to the irradiation increased the radio sensitivity of the membrane -SH groups.

Haemolysis was observed by Kollmann et al (1969) after doses of between 160-500 Gy and this is accompanied by a 57% loss of sulphydryl groups. Such haemolysis occurred in isotonic NaCl and was prevented or reduced by suspension in isotonic choline chloride or hypertonic NaCl or KCl. Prior to haemolysis in isotonic Nacl, the cells rapidly lost K^+ and accumulated Na^+. Although irradiated suspensions of cells in choline chloride did not haemolyze, they lost K^+.

The nature of the membrane injury in irradiated human erythrocytes has been studied by Shapiro and Kollmann (1968) who showed that papain increases the number of surface sulphydryl groups and increased the radiation induced sodium accumulation, whereas trypsin and neuraminidase which do not alter surface sulphydryl groups failed to elicit such a radiation effect.

Although the dose effect curve for K^+ loss from irradiated erythrocytes of man, calf, chicken and rate were all similar (Myers and Bide, 1966), those for haemolysis exhibited more variation from one species to another. Calf erythrocytes, which normally have a low potassium content, appeared to be the most sensitive to radiation induced

haemolysis.

Although K^+ loss and Na^+ gain seems to be a general effect of X-irradiation of cells, with sufficient doses, Shapiro and Kollmann (1968) showed that rubidium and caesium accumulation were also increased with radiation but calcium, strontium,chromate and phosphate fluxes were not.

In a specific study of the mechanism of the effect of ionizing radiation on Na^+ uptake by human erythrocytes, Shapiro et al (1966) showed that the amount of K^+ lost and Na^+ taken up by cells was linearly related to radiation dose although sodium uptake was slightly greater than potassium loss. The uptake of Na^+ and the loss of K^+ at 20 Gy was almost twice that of the unirradiated control. They also showed that fluoride, an inhibitor of glycolysis, and ouabain, an inhibitor of active transport did not change this radiation effect.

In searching for the cause of the permeability changes bringing about ionic changes it has been shown that intracellular factors such as glucose utilization and lactate production (Cividalli (1963), ATP production (Sheppard and Stewart, 1952) and glutathione level and stability (Cividalli, 1963 and Myers and Bide, 1966) do not appear to be involved. It would appear that most radiation induced changes are attributed to increased ion movements resulting from damage to membrane structure (Shapiro et al, 1966 and Myers and Bide, 1966).

The work in this study has demonstrated major changes in permeability of erythrocytes produced by organic mercurial compounds which bind to the membrane protein sulphydryl groups. Ionizing irradiation, its protection by glutathione and sensitization by NEM, have often implicated sulphydryl groups as being particularly radiosensitive and Cividalli (1963) hypothesized that sulphydryl groups may be critical sites in the erythrocyte membrane that are damaged by irradiation. As pointed out earlier, Sutherland et al (1967) has showed that X-irradiation produces an increase in Na^+, K^+ and choline movement, cell volume increase and haemolysis; all symptoms characteristic of those produced by the sulphydryl reagent pCMBS. Cividalli (1963) on the other hand failed to demonstrate an increase in either cell volume (haematocrit) or the percentage of cell water of irradiated erythrocytes and similarly could not detect any increase in auto-haemolysis of irradiated whole blood. They explained their findings based on the work of Bucksbaum and Zirkle (1949) who irradiated the large nucleated erythrocytes of Amphiuma and observed shrinking and later swelling of the erythrocytes before haemolysis.

Recently Dix et al (1985) has used the technique of target inactivation in an effort to determine the nature and molecular weight of water and urea transporters in human red cells. By freezing cells at -50°C in cryoprotectant solution, irradiating with 1.5 Mev electrons and then thawing, washing and assaying for osmotic water and urea transport, it was found that red cell urea transport

was inactivated by radiation (0-40 KGy) with a single target size of 469 $\pm$ 36 KDa. Water transport was not inactivated by radiation but in fact showed a slight increase with dose. The inhibitory potency of 2.5 mM pCMBS on human cells is decreased from 86% to 4% over a 0-20 K Gy dose. Because the water transport is not inactivated by radiation Dix's results do not appear to support a large molecular weight protein pathway for its transport and they have suggested passage through low molecular weight proteins or even lipid. Because the pCMBS-binding site, which regulates water flow is inactivated with radiation, they also concluded that urea transport is mediated by a specific, high molecular weight protein and does not support the hypothesis that a band 3 dimer (190 K Da) mediates both red cell osmotic water and urea transport.

When human erythrocytes are incubated at 37°C in the presence of glucose after radiation exposure, the radiation-induced permeability changes can be partly reversed. Sutherland and Pihl (1968) have reported that this recovery amounted to 60% of the -SH groups which had disappeared on irradiation, and corresponds closely to the amount of dissulphide formed.

Recovery of potassium ions during incubation at 37°C could also be demonstrated with irradiated erythrocytes from man and chicken as well as those from the rat (Myers and Bide, 1966). Interestingly a concentration of 0.01 mM ouabain, as compared to a normal experimental level of 1 mM for rat

cells was sufficient to block the recovery, perhaps indicating the increased accessibility of the enzyme site to the drug.

6.2 MATERIALS AND METHODS

All materials and methods employed in this chapter are as described in the previous chapters (3, 4 and 5) except for the following.

6.2.1 Irradiation procedures

Erythrocyte suspensions were irradiated with a Gravatom Fixed Cobalt-60 source at an exposure rate of 5.81 Gy/min. Irradiation was carried out at 9-10°C. In some experiments, the erythrocyte suspension was either out gassed with N_2 for 10 minutes before irradiation or oxygen was bubbled continuously during irradiation.

6.2.2 Determination of the Dose Rate of Gravatom Fixed Cobalt-60 source

The dose rate of the Gravatom Fixed Cobalt-60 source was determined by means of a Fricke ferrous ammonium sulphate dosimeter as described by Vereshchinskii and Pikaeu (1964). The ferrous sulphate system is the most frequently utilized dosimeter in radiation chemistry and commonly employed for X-rays, γ -radiation and electron radiation.

It consists of an aqueous solution of 1.1×10^{-3} - 5.1×10^{-3} M ferrous sulphate in 0.4 M sulphuric acid containing 10^{-3} M NaCl and saturated with air.

Procedure:

20 ml of freshly prepared ferrous sulphate in a suitable glass irradiation vessel is irradiated. At 15 minute intervals, 3 ml samples are transferred to quartz cuvettes for optical density measurements at a wavelength of 302 nm (CECIL 272 UV-spectrophotometer). On each occasion, the reading of the irradiated sample was corrected for the amount of ferric ion present by reference to the unirradiated solution (fig A).

The dose rate was calculated from the G-value of 15.5 for the oxidation of ferrous ion to ferric ion. (G-value is the number of ions or molecules formed when the system has absorbed 100 ev of ionizing radiation energy).

The basic relationship (Vereshchinskii and Pikaeu, 1964) is:

$$\text{Energy abosrobed} = \frac{OD \times 6.023 \times 10^{20} \times 1.602 \times 10^{-12}}{E.d.\ G(Fe^{3+}).1.100} \text{ Gray}_{\text{min}^{-1}}$$

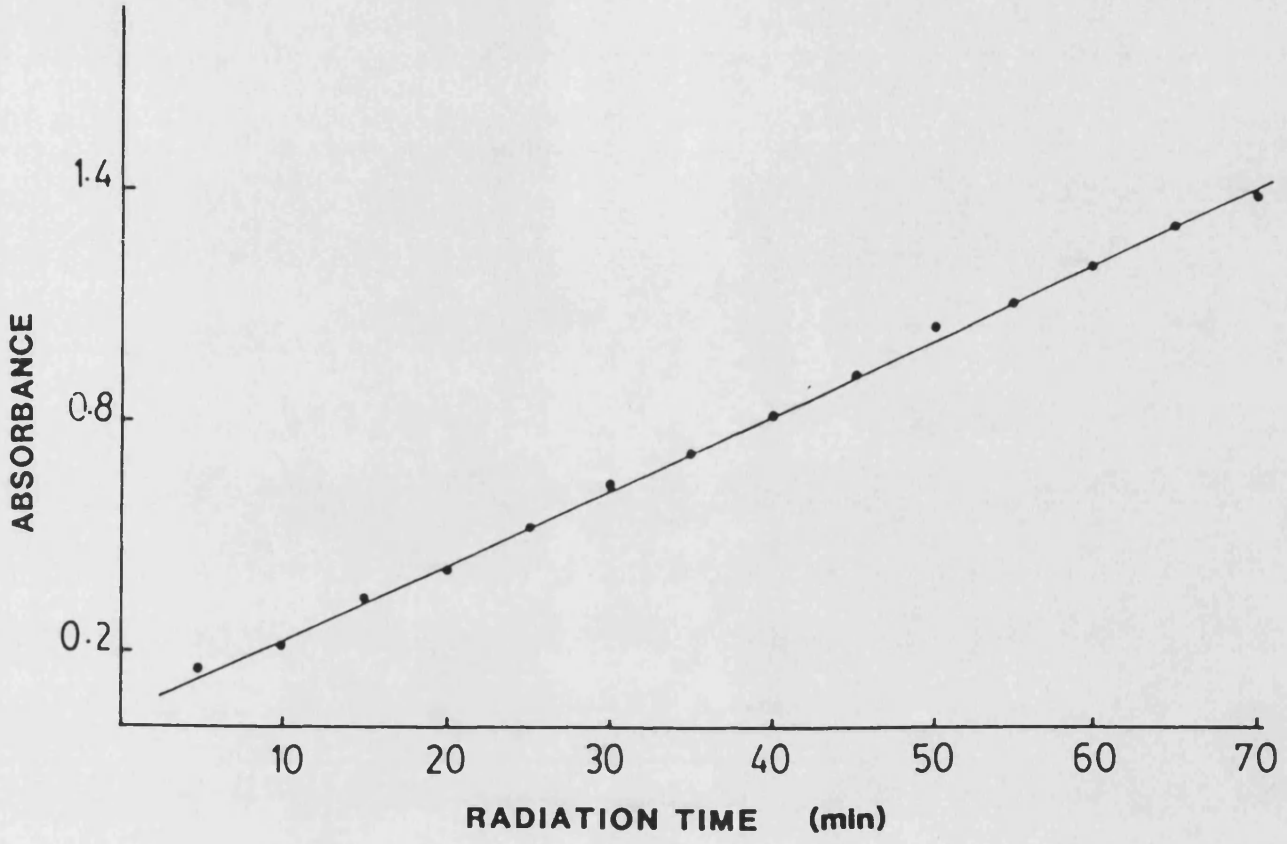
where E = Molar extinction coefficient of Fe^{3+} standard (2174 liter Mol^{-1} cm^{-1} at 24°C)

d = pathlength in cm (1cm in this case)

l = density of solution (1.024 g cm^{-3})

and G (Fe^{3+}) = 15.5 per 100 ev.

Fig A



Calibration curve for the Fricke Dosimeter relating UV absorbance against radiation dose.

6.3 RESULTS

6.3.1 Effect of Radiation on the Membrane Sulphydryl groups of human and eel Erythrocytes

The effect of gamma radiation on the loss of human membrane sulphydryl groups is illustrated in figure I . The total dose to an erythrocyte suspension (9% Haematocrit) was delivered between 40-400 Gy at 10°C and the measurement of the remaining sulphydryl groups carried out one hour post-irradiation by ^{203}Hg - pCMBS binding.

Approximately 58% of membrane -SH groups are lost at doses of 120 Gy. The disappearance curve is biphasic with a break at 120 Gy . The calculated D_{37} below and above the break were respectively 360 Gy and 274 Gy with a mean overall D_{37} between 40-412 Gy of 232 Gy . These findings show that, in general, the -SH groups of human cells are very radiosensitive.

Extrapolation of the second phase of the graph to zero dose shows that 30% (1.9×10^{-18} mol $cell^{-1}$) of the total number of membrane sulphydryl groups are the most radiosensitive.

Figure II shows that,like human cells, the -SH groups of eel erythrocyte membrane decreased as the radiation dose increases. Again as for human cells the disappearance curve does not follow a single exponential function and is biphasic with (a) an initial rapid loss of membrane -SH

groups followed by (b) a slow disappearance. The discontinuity of the -SH disappearance curve was noticed at 120 Gy at which approximately 23% of the total eel membrane -SH groups have been lost.

These results also suggest the presence of two classes of membrane -SH groups with respect to their radio-sensitivity. To test this hypothesis the values of the second portion of the curve were substracted from the composite curve to produce two discrete lines. From the slopes of these resulting curves the relevant D_{37} doses were calculated to be 490 and 1351 Gy .

Extrapolation of the straight portion of the curve to zero dose shows that the radio sensitive fraction of membrane sulphydryl groups constitute about 21% (8.7×10^{-18} mol $cell^{-1}$) of the total number.

Fig I and II

Effect of radiation dose on the number of membrane sulphydryl groups plotted as a logarithm survival curve.

Fig I for human

Fig II for eel

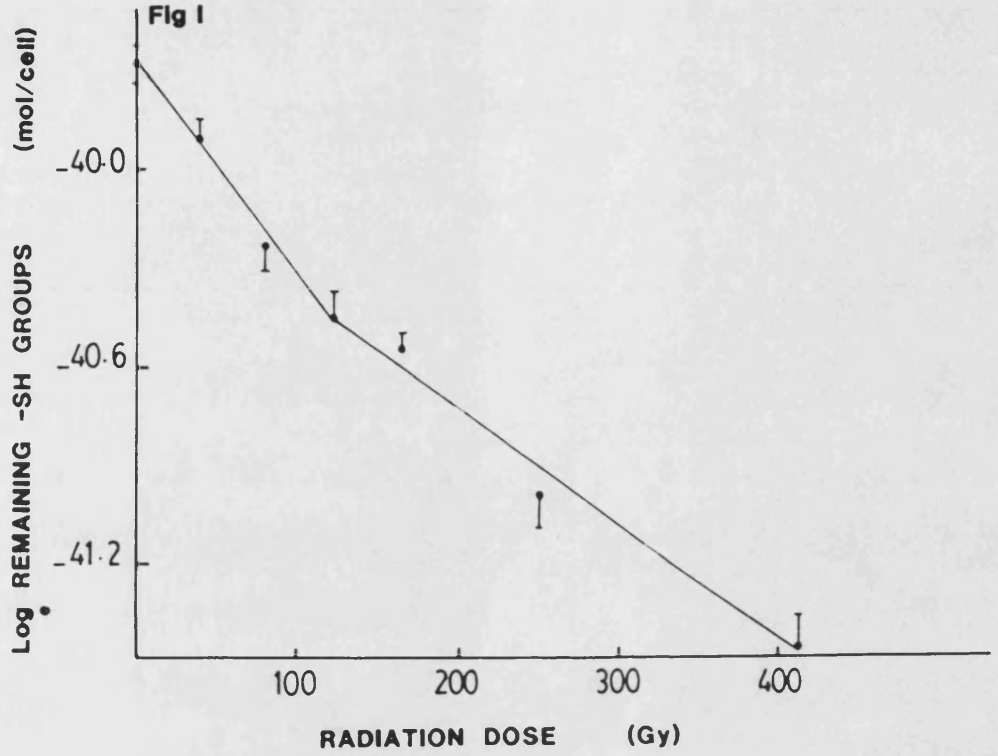


Fig I
Log REMAINING -SH GROUPS (mol/cell)
-40·0
-40·6
-41·2
100
200
300
400
RADIATION DOSE (Gy)

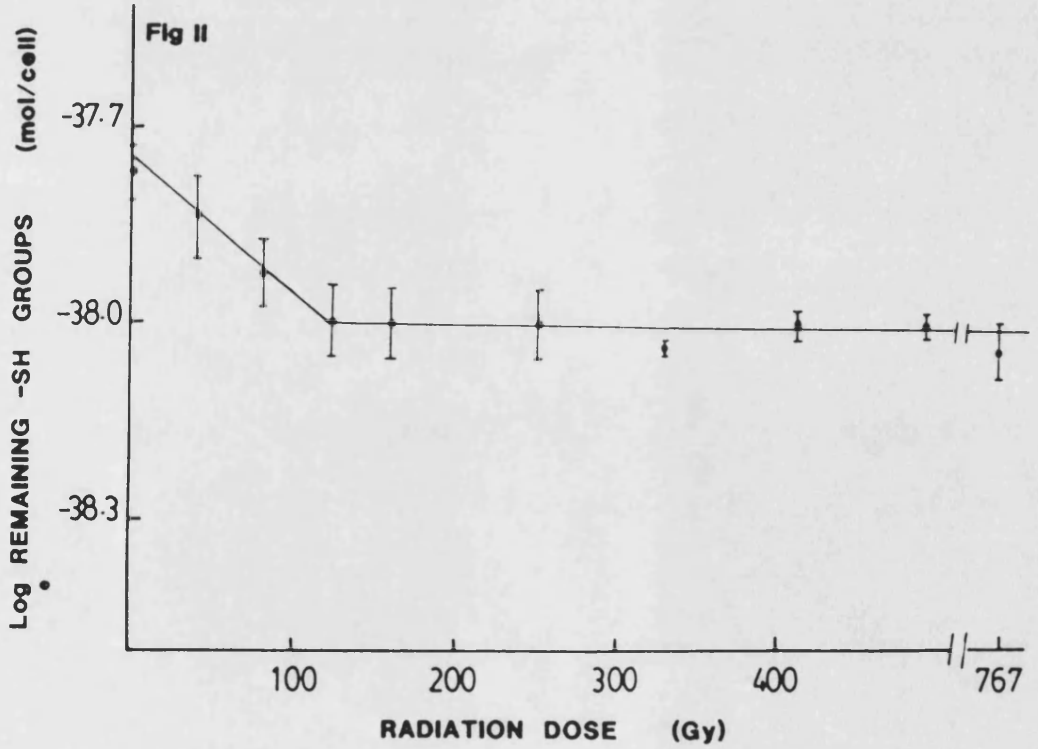


Fig II
Log REMAINING -SH GROUPS (mol/cell)
-37·7
-38·0
-38·3
100
200
300
400
767
RADIATION DOSE (Gy)

6.3.2 Effect of Radiation on Haemolysis Rate

The effects of radiation on the relative rates of haemolysis due to osmotic water flow across eel erythrocyte membranes were studied at doses between 40-412 Gy and the data shown in figure III.

In nitrogen, 1 hr post irradiation incubation at 12°C, the relative rates of haemolysis are elevated with radiation dose, while in the presence of O_2 there is a decreased rate of osmotic haemolysis.

A significant decrease in the rates of osmotic haemolysis relative to control was also recorded 24 hrs post irradiation indicating that their increased permeability may be a short lived phenomenon.

At 3 hours post irradiation for cells in nitrogen, the addition of 1 mM pCMBS produced an insignificant increase in the rates of osmotic haemolysis relative to 1 hr post irradiation suggesting that pCMBS binding sites allowing water flow are no longer effective on the membrane.

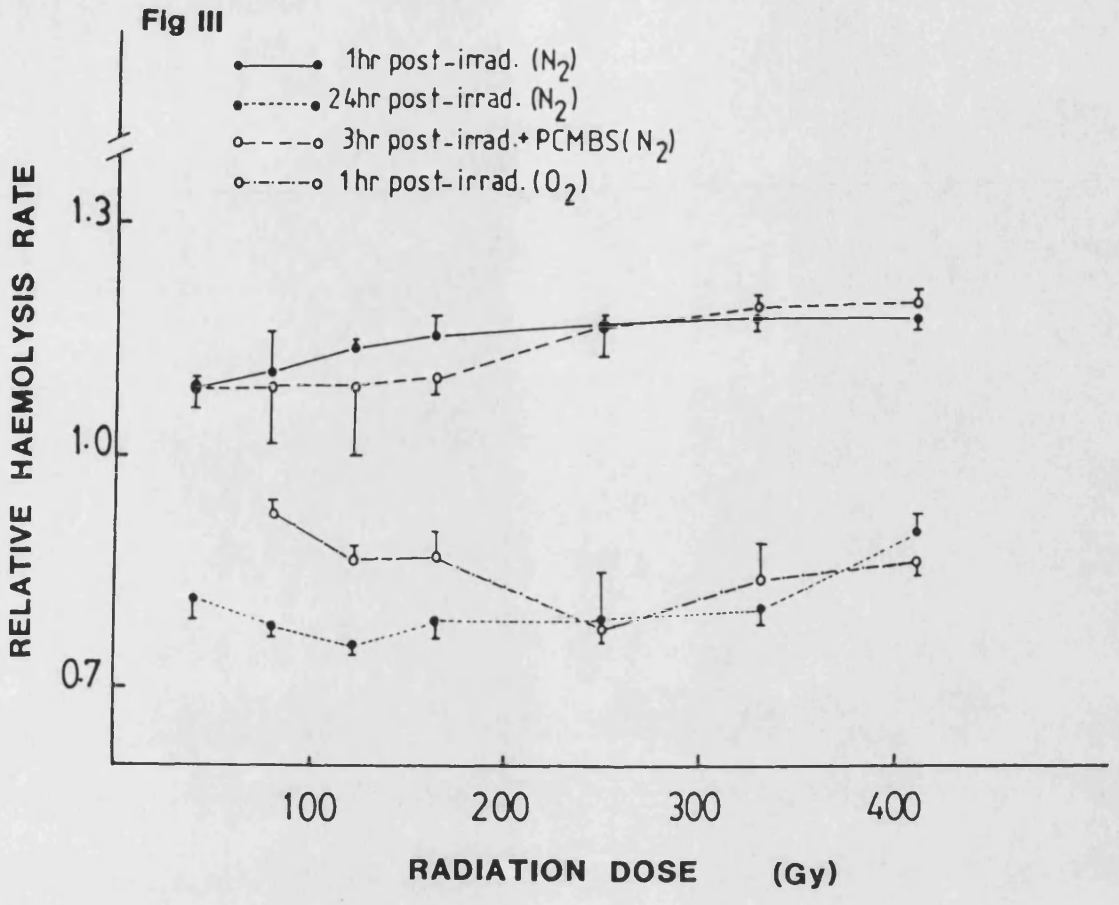


Fig III Effect of radiation and pCMBS treatment on the relative haemolysis rate of eel erythrocytes.

Figure IV shows a logarithmic plot of rates of osmotic haemolysis of eel cells against radiation dose at 1 hour post exposure in the absence of oxygen (N_2 bubbled before irradiation). By inspection of the graph and the correlation coefficient its clear that a two line is better than a one line fit with a junction around 162 Gy suggesting two populations of water controlling sites. It is also clear that the rate of osmotic flow of water varies markedly with low doses up to 162 Gy followed by little change at higher doses.

In Figure V, the same relationship was observed in the case of human erythrocytes with a break at 120 Gy . Like the eel erythrocytes, the osmotic water flow rates varies significantly at low doses of radiation below 120 Gy , with little change at higher doses.

Fig IV and V

Logarithmic plot of the rate of haemolysis against radiation dose at 12°C measured 1hr post irradiation.

Fig IV for eel erythrocytes

Fig V for human erythrocytes

Fig IV

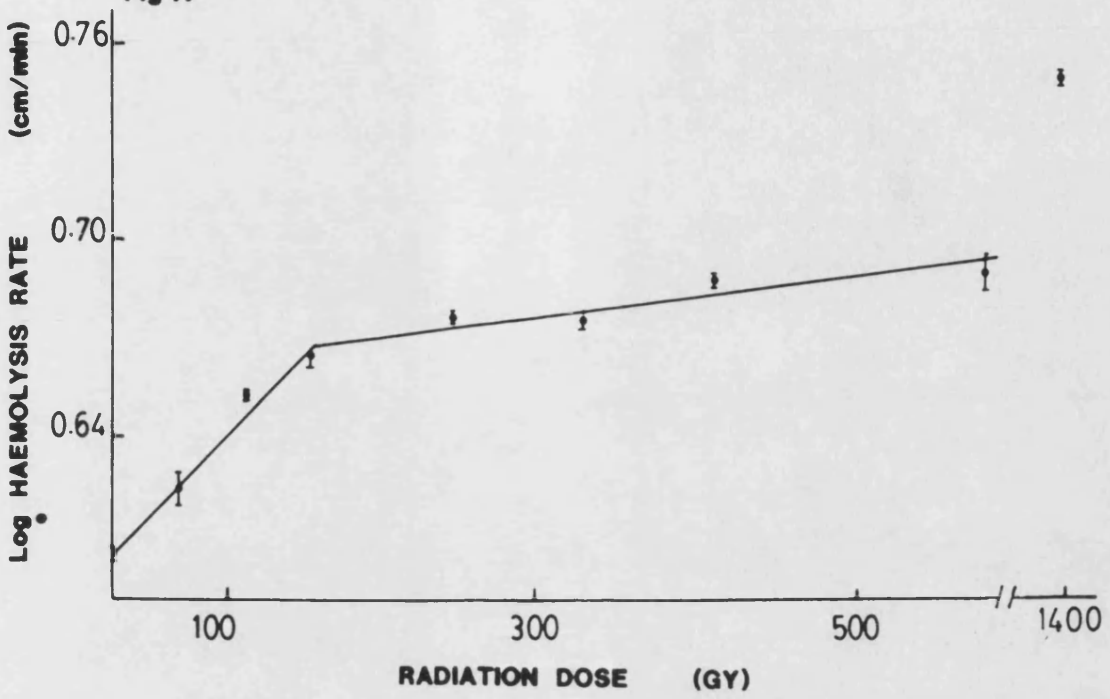
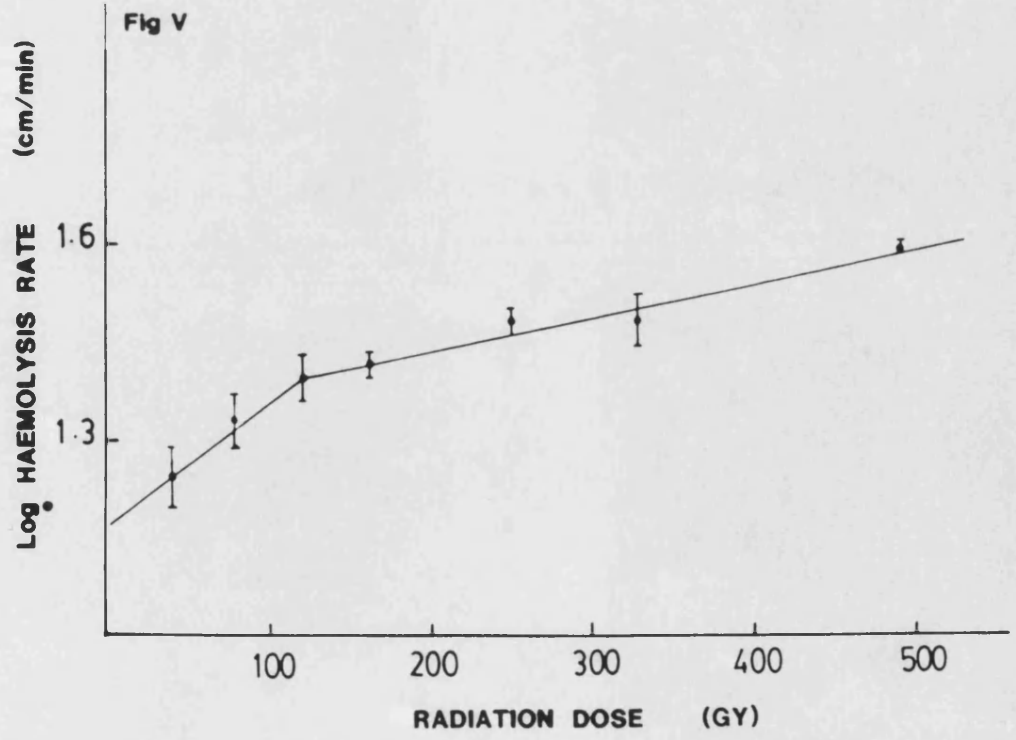

Log HAEMOLYSIS RATE (cm/min)
0.76
0.70
0.64
100
300
500
1400
RADIATION DOSE (GY)

Fig V


Log HAEMOLYSIS RATE (cm/min)
1.6
1.3
100
200
300
400
500
RADIATION DOSE (GY)

As shown previously (figure III), when eel erythrocytes were irradiated at 12°C in the absence of O_2 there was an inhibition of osmotic flow when measured as the relative rate of haemolysis 24 hours post irradiation compared to that at 1 hr post exposure. This observation was extended (figure VI) by recording the percentage of cell lysis with dose at 24 hrs (human and eel) and 6 days (eel). It is observed that the haemolysis of eel erythrocytes at zero dose is 38% at 6-days and with increasing doses of radiation the haemolysis level decreases.

In the case of human erythrocytes kept under similar conditions approximately 8% haemolysis occurred at zero dose after 24 hrs rising to 16% at 480 Gy.

In the presence of O_2 during irradiation, eel erythrocytes started to lyse at 160 Gy and the % haemolysis increased with increasing dose.

It should be noted that, unlike eel cells, human cells suffered total haemolysis within 48 hours.

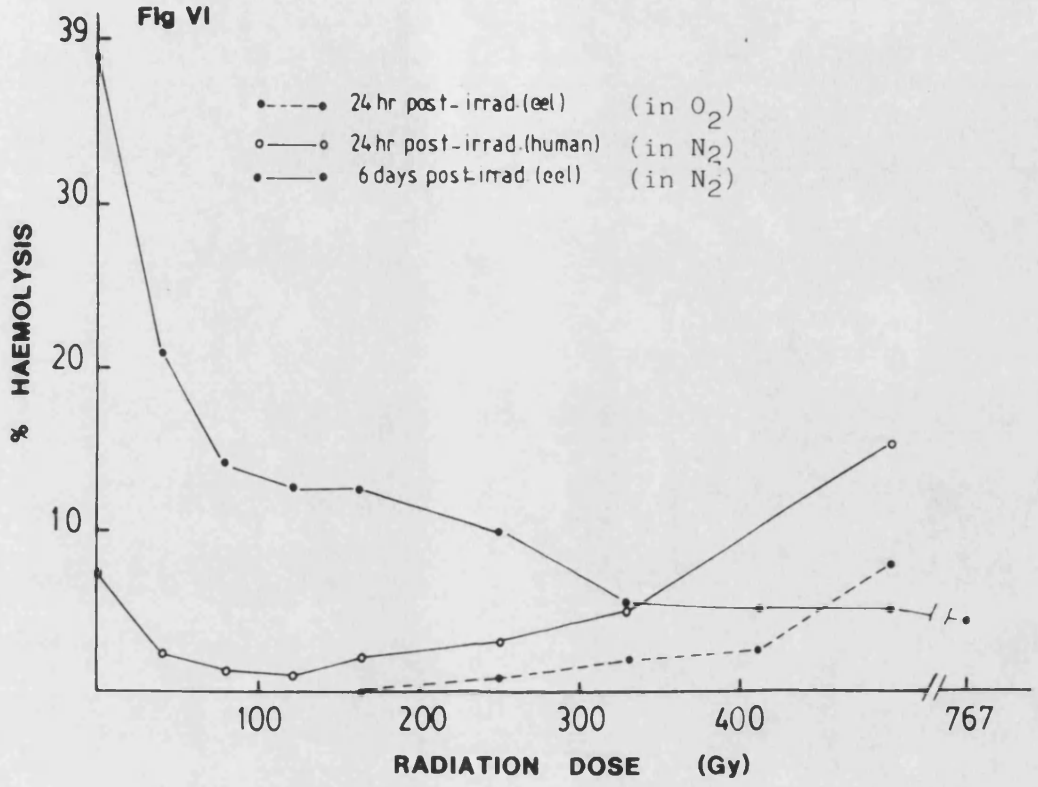


Fig VI Change in percentage haemolysis with radiation dose of eel and human erythrocytes measured 24 hrs and 6-days post irradiation (temp 12°C).

6.3.3 Effect of Radiation on Ion and Water Content of Eel and Human Erythrocytes

a) Eel data

To further elucidate the effect of radiation on the natural barrier for water and ion movement, a range of doses from 0-1400 Gy was applied to whole cells at 12°C and the water, Na^+, K^+ and Cl^- contents measured 24 hour post irradiation.

Figure VII shows the cellular water content of eel erythrocytes in the presence and absence of oxygen. In the absence of oxygen, there was a marked decrease in water content relative to the experimental control (kept under the same condition) which itself showed an elevated water content (3200 ml Kg^{-1} dcs) compared to the normal control (2100 ml Kg^{-1} dcs). The large difference between the two controls is obviously due to the lack of oxygen and suggests that, unlike human cells, eel erythrocytes, contining mitochondria use the Krebs cycle for volume control. High radiation doses (1400 Gy) showed higher water content over both controls.

In the presence of oxygen during irradiation, eel erythrocytes showed a decreased water content below the experimental control up to 330 Gy then recorded a slight increase of water content with some haemolysis (8%) at higher radiation doses.

The level of Na^+ ions in eel erythrocytes, measured 24 hrs post irradiation at 12°C is illustrated in figure VIII. Up to a dose of 230 Gy, the levels are maintained below those of both the experimental control (kept 24 hrs at 12°C) and the normal control. At higher doses there then follows a small increase to reach the normal control level. Again at 1400 Gy there is significant increase of cellular Na^+ content compared to the control.

The presence of oxygen during irradiation, appeared to have only minimal effect on the cells compared to those irradiated in its absence. Cellular Na^+ content increases with increasing doses to reach the normal content 24 hrs post exposure to 480 Gy.

At zero radiation dose, Figure IX shows a general loss of K^+ content of eel red cells after 24 hrs incubation at 12°C. The loss is considerable in the case of cells bubbled with N_2 before exposure. With irradiation in the absence of oxygen, although there was a significant loss of cellular K^+ 24 hr post exposure, the levels were higher than those of the experimental control kept under the same conditions.

In the presence of oxygen during irradiation, there is also a K^+ loss after 24 hrs post exposure, which is significantly below both the experimental and normal controls.

Figure X represents the cellular Cl^- content of eel red cells measured 24 hrs post exposure to doses of between 0-490 Gy at 12°C. At zero dose in the absence of oxygen there is a significant Cl^- uptake whereas with O_2 there is little difference over the normal control levels.

The irradiation of eel erythrocytes in the absence of O_2 inhibited Cl^- uptake compared to the experimental control, while in its presence there was a significantly decreased level. The decreased Cl^- content is significant at 240 Gy and reached the control limit at doses of 490 Gy.

Fig VII to X

Effect of radiation on water, Na^+, K^+ and Cl^- contents of eel erythrocytes at 12°C 24 hr post irradiation.

Fig VII **water content**

Fig VIII **Na^+ content**

Fig IX **K^+ content**

Fig X **Cl^- content**

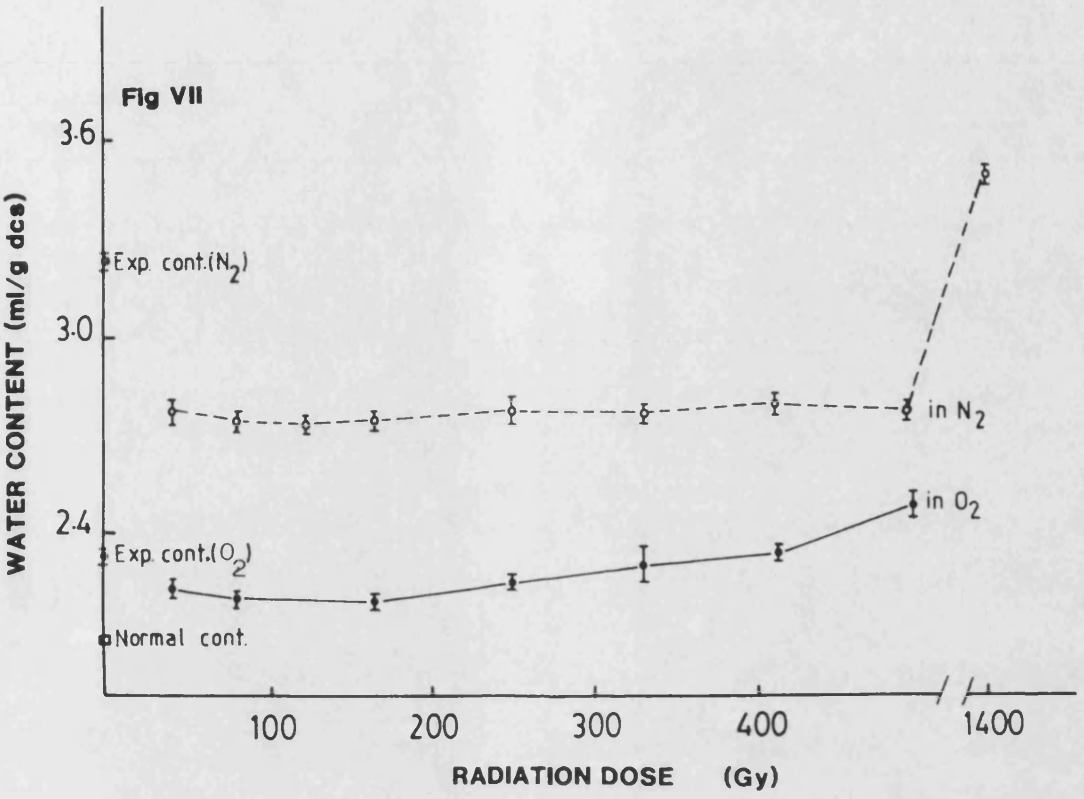


Fig VII
3·6
3·0
2·4
WATER CONTENT (ml/g dcs)
Exp. cont.(N2)
Exp. cont.(O2)
Normal cont.
in N2
in O2
100
200
300
400
1400
RADIATION DOSE (Gy)

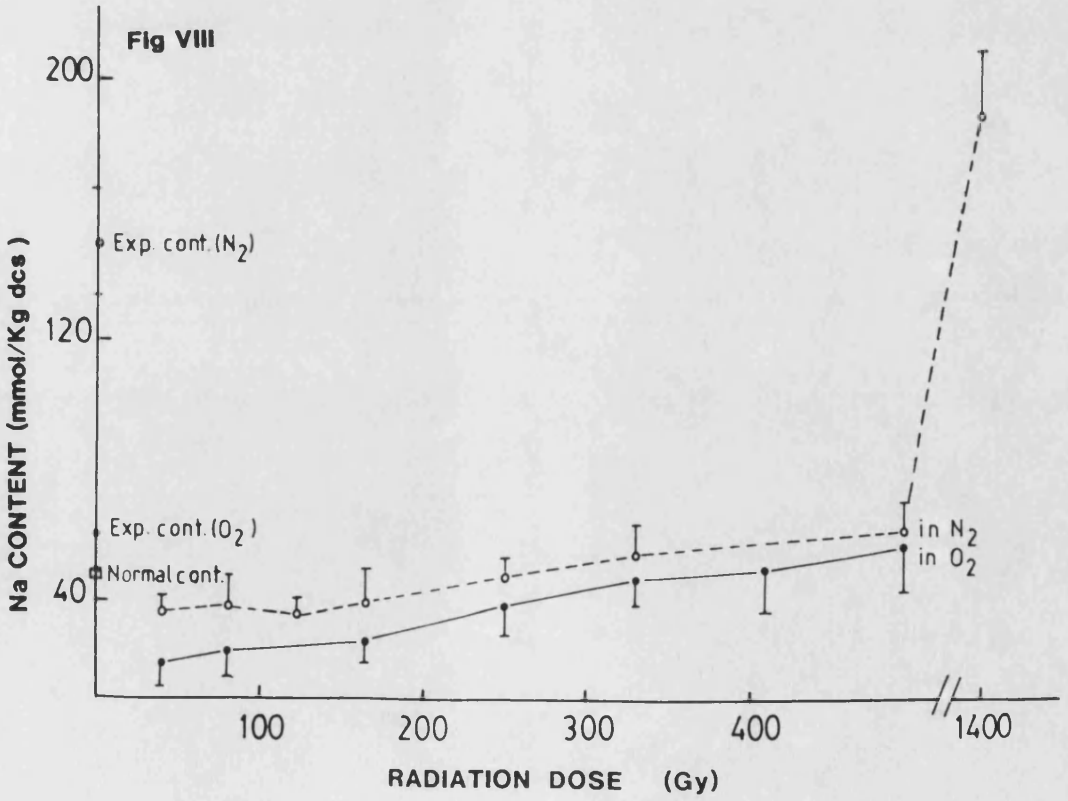


Fig VIII
200
120
40
Na CONTENT (mmol/Kg dcs)
Exp. cont.(N2)
Exp. cont.(O2)
Normal cont.
in N2
in O2
100
200
300
400
1400
RADIATION DOSE (Gy)

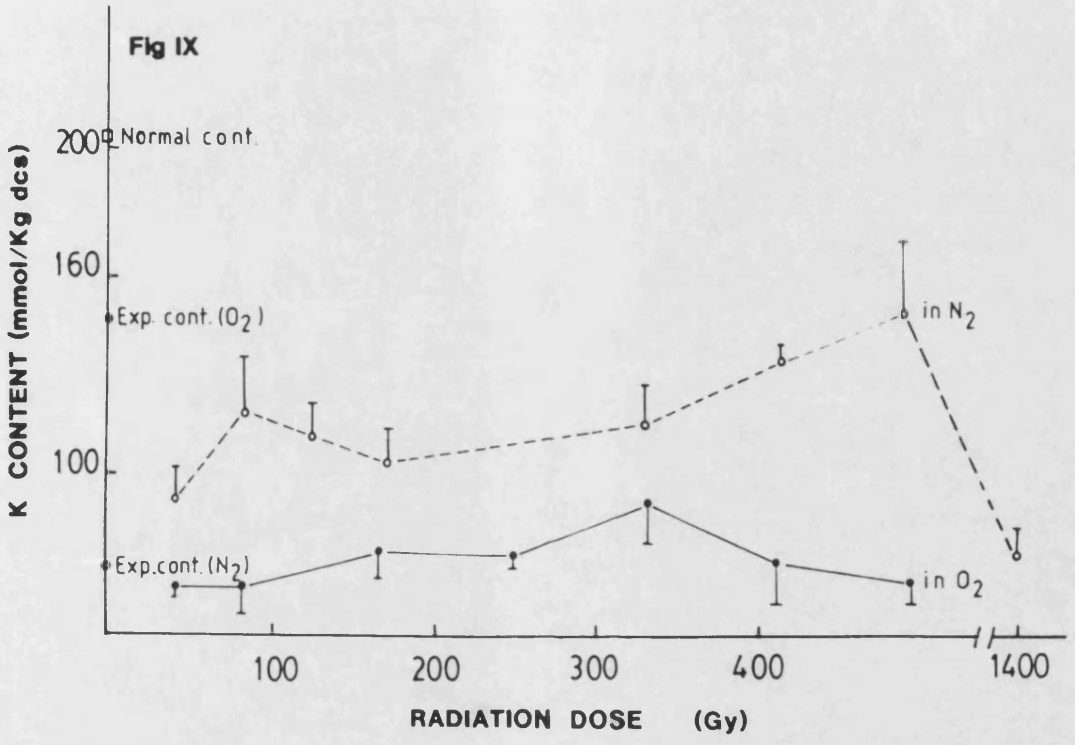


Fig IX
200
160
100
Normal cont.
Exp. cont. (O_2)
Exp.cont. (N_2)
in N_2
in O_2
K CONTENT (mmol/Kg dcs)
100
200
300
400
1400
RADIATION DOSE (Gy)

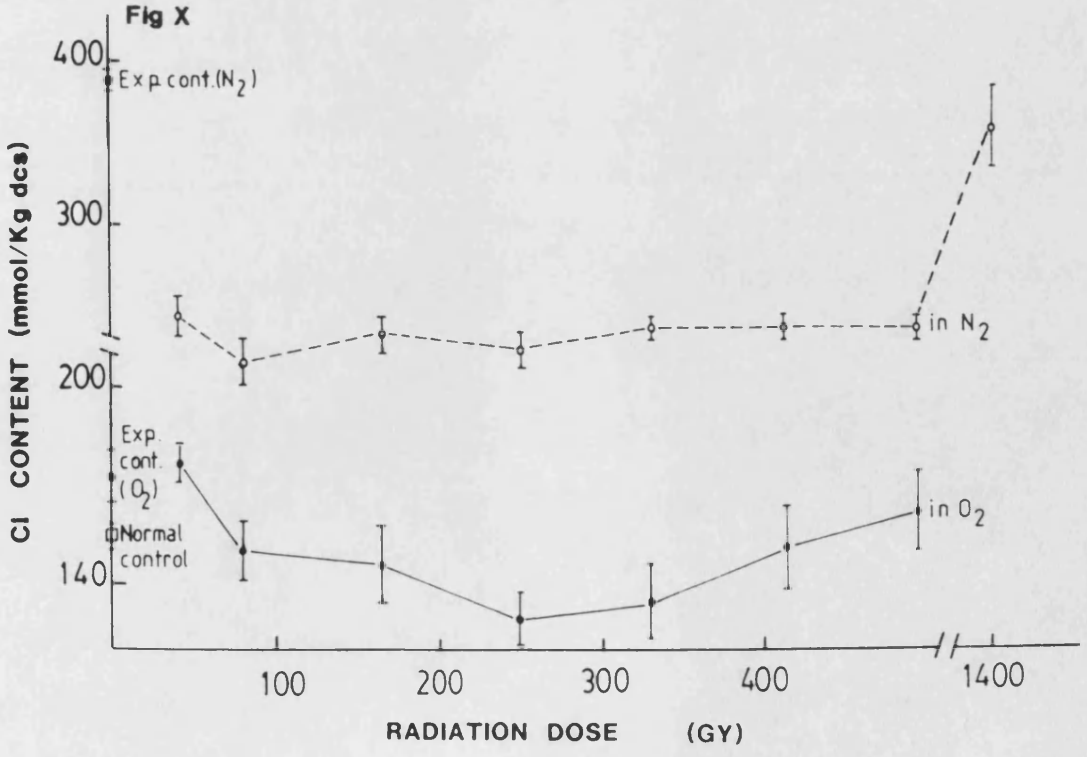


Fig X
400
300
200
140
Exp.cont.(N_2)
in N_2
Exp. cont. (O_2)
Normal control
in O_2
Cl CONTENT (mmol/Kg dcs)
100
200
300
400
1400
RADIATION DOSE (GY)

b) Human data

The effect of gamma radiation on water and ion movement in human erythrocyte cells suspended in buffered saline solution, pH 7.4, at 12°C was studied 24 hours post irradiation in the absence of O_2 and the results shown in figs XI to XIV. The data show a linear increase in water, Na^+ and Cl^- contents paralleled with K^+ loss as the dose of radiation increase. The uptake of sodium and the loss of potassium at 40 Gy were almost twice that of the unirradiated control without significant increase in water content.

The experimental control in the absence of O_2 showed a small increase in water, Na^+ and Cl^- contents coupled with a small K^+ loss after 24 hr incubation.

Fig XI to XIV

Effect of radiation on water, Na^+, K^+ and Cl^- contents of human erythrocytes at 12°C 24 hrs post irradiation.

Fig XI **water content**

Fig XII **Na^+ content**

Fig XIII **K^+ content**

Fig XIV **Cl^- content**

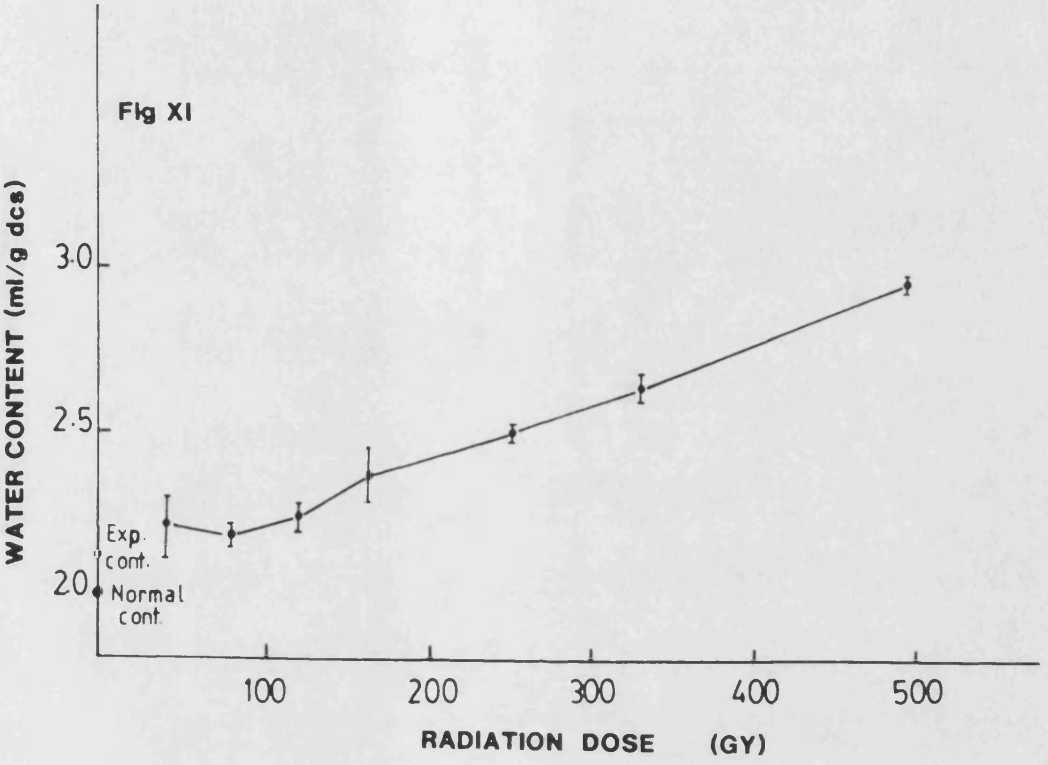


Fig XI
WATER CONTENT (ml/g dcs)
3·0
2·5
2·0
Exp. cont.
Normal cont.
100
200
300
400
500
RADIATION DOSE (GY)

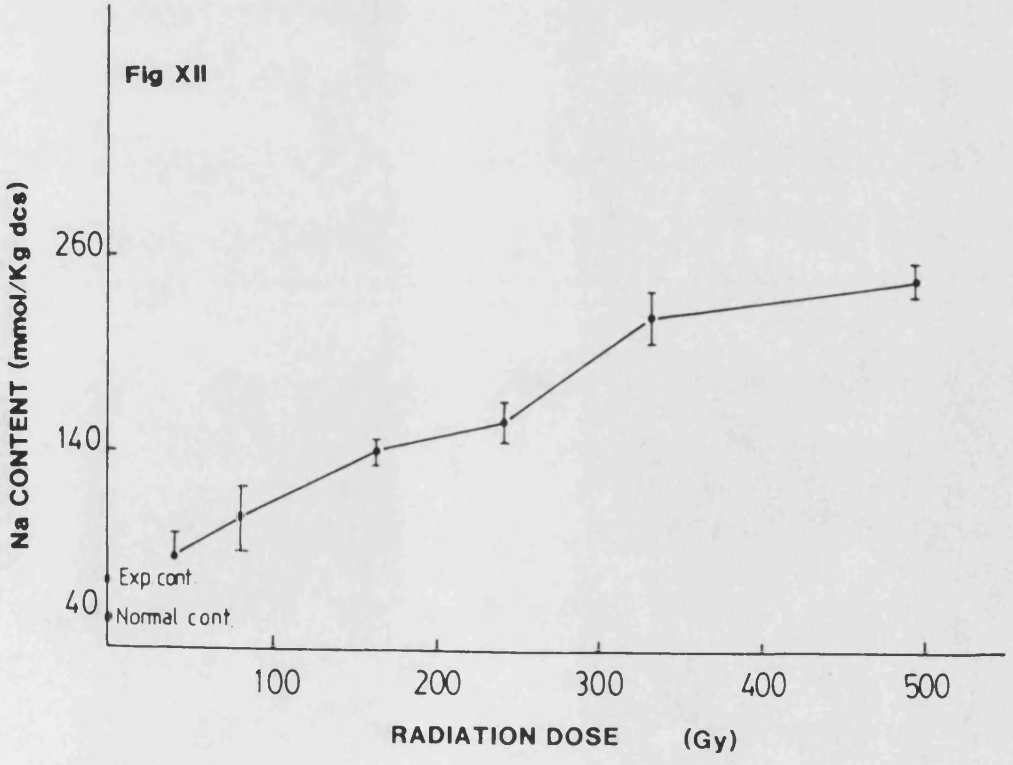


Fig XII
Na CONTENT (mmol/Kg dcs)
260
140
40
Exp cont.
Normal cont.
100
200
300
400
500
RADIATION DOSE (Gy)

Fig XIII

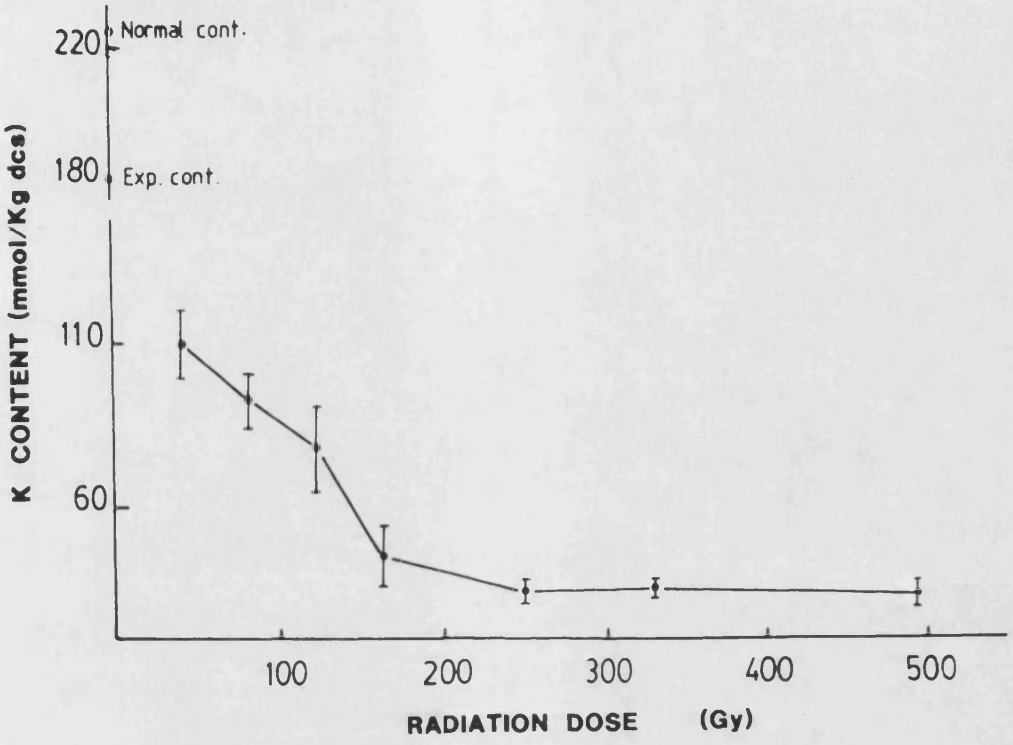
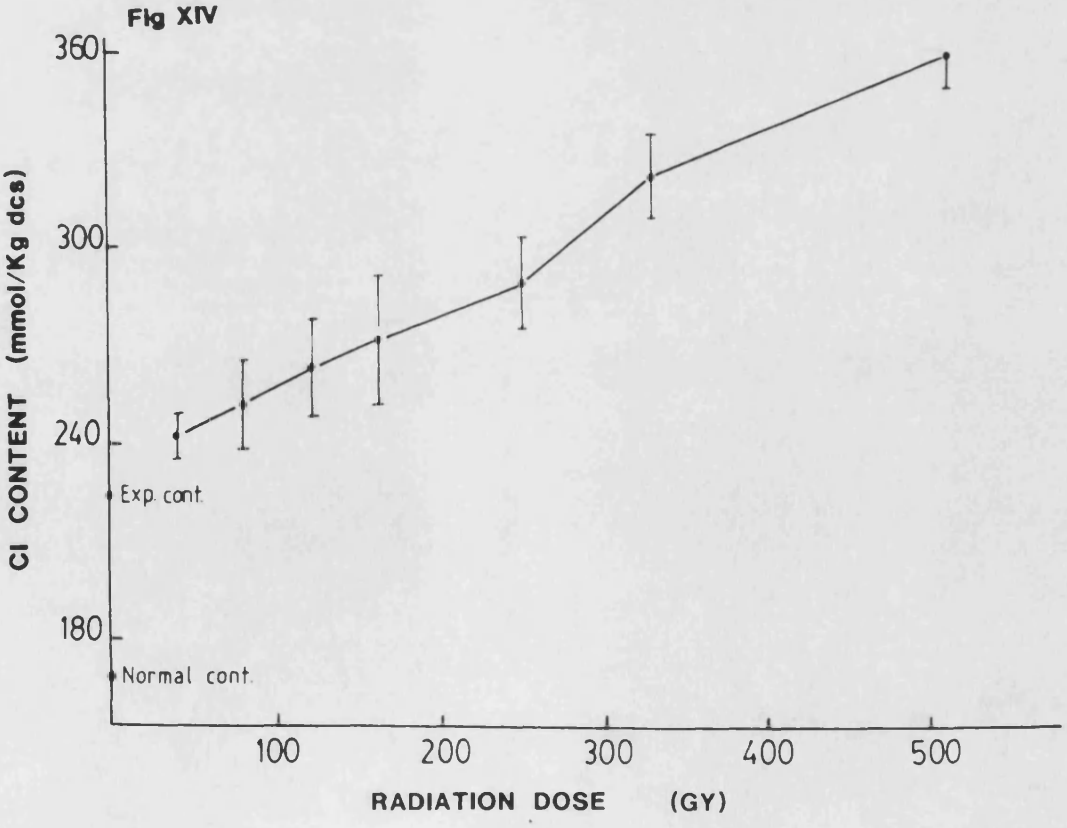


Fig XIV



6.3.4 Reappearance of Membrane -SH groups

In the typical ^{203}Hg labelled pCMBS experiment (Chapter 4), the data in fig XV shows the reappearance of membrane -SH groups when eel erythrocytes are incubated at 12°C in the presence of glucose for 24 hrs after exposure to various doses of radiation. The recovery is complete and recorded higher membrane -SH groups to that at zero dose.

Sutherland and Pihl (1968) reported that the unirradiated human cells lose 10% of the -SH groups in the course of 6 hrs incubation. The human erythrocyte data treated in the same way as eel cells are illustrated in figure 15 and table 1. It is evident that there is reappearance of some of membrane -SH groups after 24 hours compared to 1 hr post irradiation but that it is incomplete. This may be attributed to the low temperature used during the incubation period.

Table 1: Percentage loss of Membrane -SH groups at 12°C in human Erythrocytes exposed to γ - radiation

Irradiation Dose in Gy / Lapse of time	40	80	122	162	250	410
1 hour post irradiation	21.0	44.38	52.88	58.30	73.38	83.23
24 hrs post irradiation	1.84	8.66	11.56	17.68	15.66	23.96

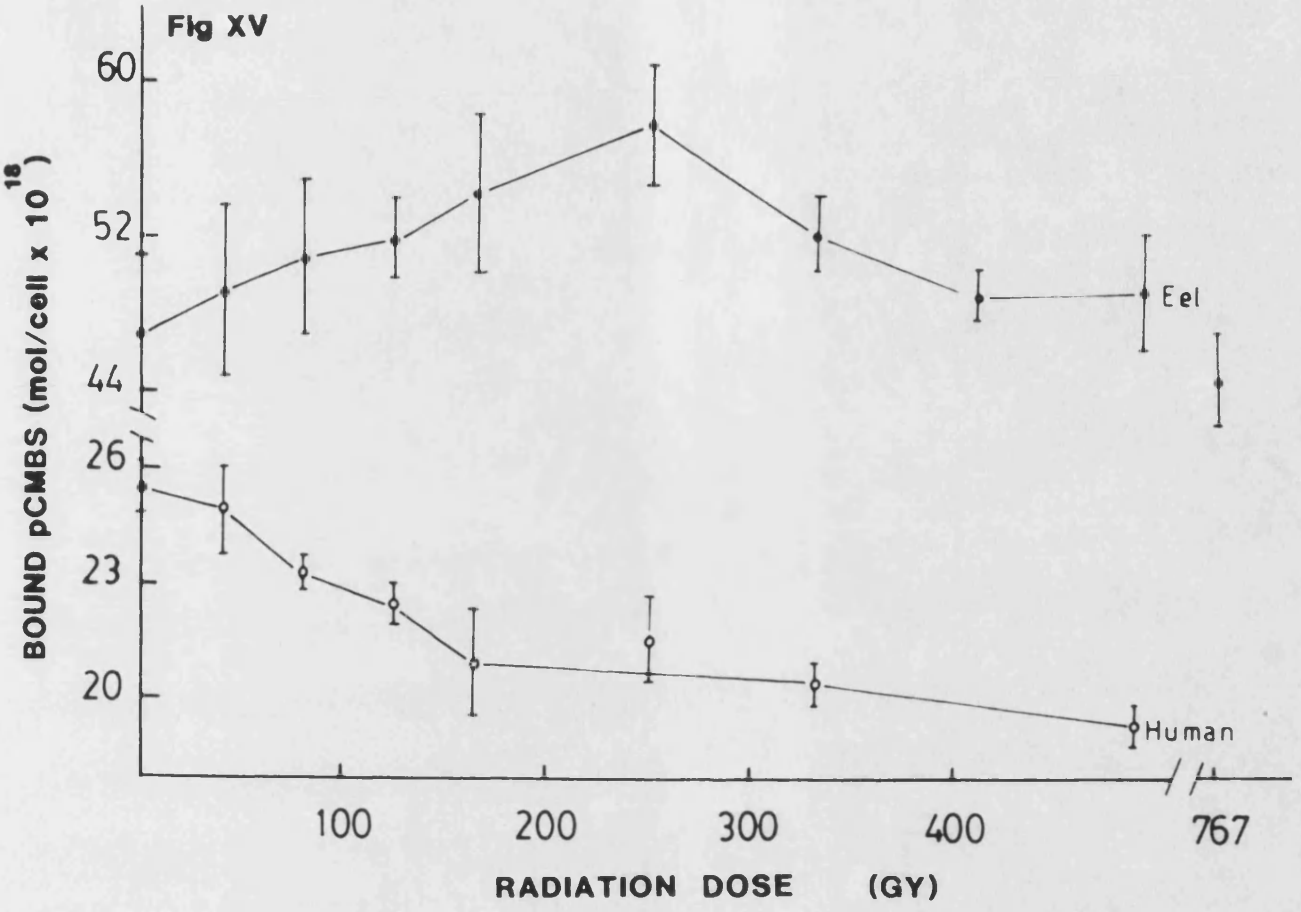


Fig XV Reappearance of membrane -SH groups of eel and human erythrocytes 24 hrs post irradiation incubation at 12°C. ^{203}Hg-pCMBS concentrations used are 3.8 x 10^{-4} M and 2.9 x 10^{-4} M for eel and human cells respectively.

6.4 DISCUSSION

It is becoming increasingly clear that damage to cellular membranes is an important factor in radiation induced cell death, (Vegt et al, 1985) and there are numerous studies showing different types of structural damage induced by ionizing radiation (Guidoni et al , 1985 and Edwards et al ,1984).

The data presented in this work confirms many of the previous observations that human erythrocytes lose their membrane sulphydryl groups after exposure to ionizing irradiation. The data also establishes that exposure of eel cells to gamma radiation (40-400 Gy) also results in a disappearance of membrane sulphydryl groups and that both eel and human erythrocytes have a mechanism for repair of this damage.

It had been concluded previously (Sutherland et al, 1967 and Sutherland and Pihl, 1968) that the radiation induced loss of sulphydryl groups was mainly due to disulphide formation and that the observed recovery of these groups was due to reduction of these disulphides.

It appears that human sulphydryl groups are more radiosensitive than those of eel with 58% being lost in human compared to a 23% loss for eel cells after doses of radiation of 150 Gy . The human data is in agreement with those of Kollmann et al (1969).

This present study reveals that the membrane -SH groups of eel and human can be classified into two groups dependent on their radiosensitivity. From the disappearance curves (Fig I) for human, the D_{37} for the radiosensitive groups is 232 Gy in a dose range of 40-412 Gy., a value in agreement with that of Sutherland and Pihl (1968) who also showed that the less radiosensitive -SH groups were only affected by doses greater than 1000 Gy and returned a D_{37} value of 13700 Gy.

For eel erythrocytes the D_{37} was found to be 490 and 1351 Gy respectively for radiosensitive and less sensitive groups measured under the same condition as human cells.

The presence of two populations of sulphydryl groups is also in accord with the previous findings discussed in chapters 3 and 4 of this work showing two classes differing in their membrane location. This finding is further supported by the work of Shapiro et al (1966) who found that the radiosensitive groups are located on the outer surface of the cell while the others are inside the membrane. Their suggestion was based on the fact that mercaptoethylguanidine (MEG), which does not enter the cell, reverses the radiation effect when added after irradiation.

The observation that membrane -SH groups differ in their radiosensitivity is also consistent with the view that they differ in their reactivity towards various sulphydryl

blocking agents. Shapiro et al (1970) considered there to be five classes of membrane -SH groups and showed that those that react with BMHP, do not appear to be sensitive to radiation. This was based on the fact that 400 Gy did not alter BMHP binding to human erythrocytes. In the same way, Archer and Wills (1973) showed that some -SH groups were sensitive to particularly low doses of radiation (10 Gy and below) whereas others are much more resistant to 20 Gy and above. They found that irradiation with doses of 10 Gy failed to alter the pattern of NEM binding and therefore suggested that the most sensitive -SH groups to NEM are also the most radiosensitive.

In this work, the relationship between radiosensitivity and the effect of the sulphydryl blocking agent, pCMBS, was tested functionally by the addition of the mercurial to irradiated eel erythrocytes (40-412 Gy) and measuring the relative rates of osmotic haemolysis (Fig III p 218). As there was no increase in the relative rate of water flow, it would suggest no more binding of pCMBS to eel cells and shows that the radiosensitive -SH groups are the same groups that are sensitive to pCMBS. The work of Dix et al (1985) is in agreement with this suggestion, for these authors showed that the inhibitory potency of 2.5 mM pCMBS for water transport across human erythrocyte membranes is decreased from 86% to 4% over 0-20 Gy dose range.

Doses of 120 to 412 Gy did not increase the water flow rate or reduce the measurable SH groups than those of 200 Gy or below, so that some of these groups are well protected inside the membrane probably by such radioprotectors as glutathione. This finding is in accord with that of Archer and Wills (1973) who showed that 20 Gy did not, even after NEM treatment, reduce the SH levels to zero.

Post-irradiation incubation of human erythrocytes at 12°C for 24 hrs with glucose showed an incomplete reappearance of membrane -SH groups. This is consistent with the work of Sutherland and Pihl (1968) who reported that the 60% of the -SH groups lost on irradiation reappeared after incubation for 1 hr at 37°C in the presence of glucose but there was no recovery at 37°C in the absence of glucose.

In contrast the post-irradiation incubation of eel erythrocytes at 12°C in the presence of glucose showed a complete recovery and to such an extent that there was over compensation with more SH-groups appearing than before irradiation.

Such reappearances of SH groups in the presence of glucose suggests that both cell types have an active energy demanding mechanism that reduces disulphide bonds. The obvious candidate is a glutathione reductase system, an explanation based on the finding that glutathione levels were reduced immediately after irradiation but subsequently recovered on post irradiation incubation (Edwards et al, 1984). Glutathione levels in irradiated (1000 Gy) rat

erythrocytes returned to normal very rapidly at 37°C but more slowly at 4°C (Myers and Bide, 1966). It is also relevant that a radiation induced decrease of intracellular glutathione of human erythrocytes is rapidly reversed when the cells are incubated at 37°C in presence of glucose.

The incomplete reappearance of -SH groups in the case of human cells as compared with the more than complete appearance seen in eel cells at 12°C may be explained by the low temperatures involved. With 12°C being considered a normal temperature for eel cells and of course low for human cells, it would seem obvious that the recovery mechanism is operating normally in eel and subnormally in human erythrocytes. The increase in measurable -SH groups over unirradiated eel erythrocytes observed on post irradiation incubation for 24 hrs suggests that there may be a conformational change in membrane protein and/or lipid rendering more -SH groups in the membranes accessible to pCMBS.

The fact that there is only limited haemolysis in eel erythrocytes 6-days post irradiation incubation at 12°C (fig VI) compared to that in human cells 24 hrs post irradiation could be as a result of the changes occurring in the -SH groups after recovery rendering the eel membranes more haemolysis resistant. This suggestion is on a par with that observed by Lessler (1959) who found an increase in the number of irregularly shaped and cytologically abnormal cells following doses of 0.5 Gy of X-rays to

nucleated frog and Amphiuma red cells. Such abnormalities were accompanied by the production of a haemolysis resistant population of cells after 24 hrs post irradiation. Analagous results were also reported by Alpen et al (1954) who found that 24 hrs after 5 Gy of whole-body X-irradiation of rat, there was an increased resistance to osmotic haemolysis.

The results presented in this chapter show that the membrane sulphydryl groups are the major targets for radiation induced changes in Na^+, K^+, Cl^- and water permeabilities.

The data obtained for human erythrocytes confirm the observations of other workers (Shapiro et al, 1966, Sutherland et al, 1967, and Edwards et al, 1984) in that gamma irradiation causes the cells to lose K^+ and to gain Na^+, Cl^- and water faster than both experimental and normal controls. The correlation between these permeability changes and the decrease in number of membrane -SH groups after irradiation shows that -SH groups must be involved. Because in chapter 5, it was shown that the blocking of membrane -SH groups by the specific sulphydryl reagent, pCMBS, produces significant changes in ion and water permeabilities similar to those produced by radiation it is proposed that both agents act on the same controlling site (-SH groups) to produce permeability changes in the erythrocytes.

At 24 hrs post irradiation in the absence of oxygen, eel erythrocytes have a depressed K^+ and elevated Cl^- and water content compared to those of normal and experimental controls (fig VII to X p 227). Although the overall effects appear similar to those observed for human cells, the K^+, Cl^- and water level in eels remain constant between doses of 40-412 Gy (ie ion status appears to be dose independent).

The Na^+ levels are also depressed compared to both controls between 40-412 Gy. The decrease is most marked at 40 GY but with higher doses normal levels are regained, results that indicate eel erythrocytes becoming less permeable to sodium on irradiation. This is a common observation and may be compared with the report by Curran et al (1960) who found a 60% decrease in Na^+ flux across the ileum of rats exposed to doses of 20 GY.

The low Na^+ and high K^+ concentrations observed in eel erythrocytes at 24 hrs post irradiation are consistent with the observed recovery of the membrane sulphydryl groups as measured by pCMBS binding experiment.

The increased ion and water permeability observed in human experimental, non irradiated controls in the absence of O_2 (fig Xl to XIV p 231) as compared to normal controls may be because of a loss of membrane -SH groups on incubation, a fact first referred to by Sutherland and Pihl (1968) (10% loss of SH groups in 6 hours).

The increased permeabilities for Na^+, K^+, Cl^- and water across eel cells of experimental controls in the absence of oxygen compared to those of irradiated cells may well be attributed to a radiation induced recovery process, a suggestion supported by the data of fig XV showing the reappearance of eel erythrocyte membrane -SH groups. These data appear to suggest that eel erythrocytes have an active permeability controlling mechanism dictated by membrane -SH groups that operate against radiation (or any) injury. The obvious possibility is that the normal cellular pathways used for the maintenance of membrane structure, such as reduced glutathione or lysosomal enzymes, are far more effective in nucleated eel than human red blood cells.

The repair mechanisms of radiation injury also depend on the presence of glucose, a view based on the reported absence of radiation damage to cell glucose transport (Vegt et al, 1985). This is of particular interest because pCMBS is a potent inhibitor of glucose transport (Van Steninck et al, 1965 and D'Amore and Theodore, 1986) and cells are unable to repair the apparently similar pCMBS effects.

The data of fig XV (p 235) show that because of the limited reappearance of some -SH groups in human red cell membranes at 12°C, the permeability recovery is incomplete. This leads to suggestions that 1) the recovery system in eel is more effective than in human cells at the low temperature, 2) the repair in human cells is temperature dependent because complete recovery of permeability has been reported

by Sutherland and Pihl (1968) at 37°C and 3) the number of -SH groups that have reappeared (table 1) in human cells, kept at 12°C, are insufficient to restore normal permeability status.

Reference to figures VII & X for the data on non irradiated cells generates some relevant information on the normal physiological status of ion and water balance of eel red blood cells. After 24 hours under anerobic conditions the cell water content has gone from 2.1 ml g^{-1} dcs to 3.2 ml g^{-1} dcs, whereas cells in oxygen maintain approximately control levels. Volume control in these nucleated cells is obviously an aerobic process unlike the mechanism in human cells (fig XI). Also under nitrogen, eel cells lose K^+ and gain NaCl, explaining the water gain. Any volume control of hypotonically swollen cells based upon a KCl cotransport system out of the cell (Shawkat, 1984) is obviously not functional under these conditions, possibly because the internal K^+ may have reached too low a level to produce an uphill movement of Cl^- ions out of the cell. This is exacerbated by the significant movement of Na^+ ions inwards down their concentration gradient which in osmotically swollen cells appears to be blocked (Shawkat, 1984).

The presence of distinct mitochondria in these nucleated cells (plate I), supports the aerobic nature of cell processes particularly volume control and radiation damage repair which is obviously much more effective than anucleate human cells.

The similarities in the effects produced by radiation and pCMBS on Na^+, K^+, Cl^- and water permeabilities, the documented decrease in membrane -SH groups immediately post irradiation, the repair or reappearance 24 hour post exposure, the oxidation of disulphide to sulphydryl groups (Sutherland et al, 1967) and the protection by -SH containing compounds (Shapiro et al, 1966) present strong evidence for the suggestion that the membrane -SH groups are responsible for radiation induced changes in membrane permeability to ions and water.

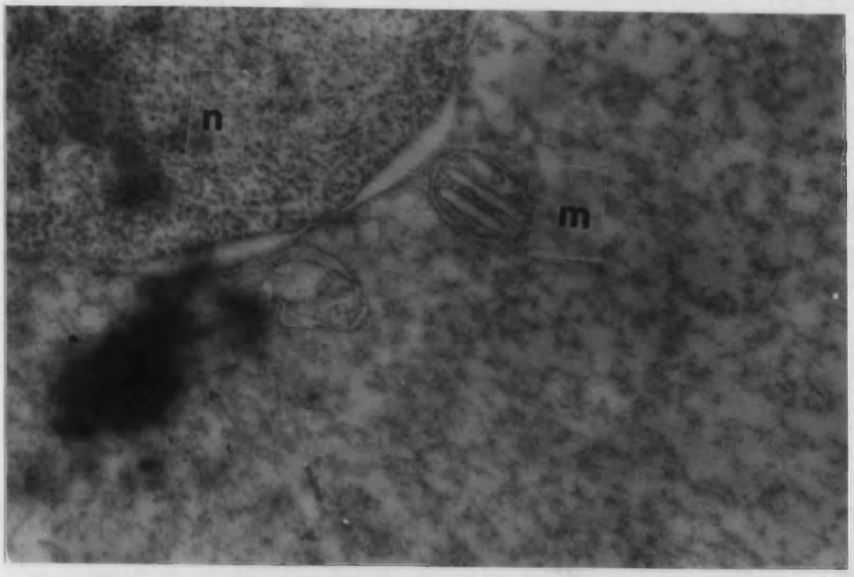


Plate I: Transmission electron micrograph of nucleus (n) and cytoplasm of eel red blood cell showing presence of mitochondria (m) x 16.600.

Summary

SUMMARY

This investigation does not support the presence of a definite thermal transition in the membranes of eel or human erythrocytes. Straight Arrhenius plots were obtained for osmotic flow in unwashed erythrocytes whilst a weak break occurred between 19-27°C in washed cells. This difference would seem to indicate the importance of plasma factors (notably SH groups) influencing membrane function and structure with respect to water transport and not a transition state change in the lipid or protein component of the membrane.

In terms of activation energy the water flow across eel red cells is similar in nature to that reported for dog erythrocytes demonstrating values close to those for bulk water viscosity. This implies that transport is viscous in character through channels offering little impedance to flow.

The activation energies for water movement across human erythrocyte membranes are lower than that for bulk water viscosity indicating a low degree of water membrane interaction. Because the value for E_a for washed cells was lower than that for unwashed cells it also strongly suggests that a plasma factor influences membrane function.

The activation energy for the water exchange time in human cells measured by the NMR T_2 technique was higher (29.8 KJ mol^{-1}) than that for self diffusion and suggests a moderately high degree of water-membrane interaction (under steady state) when compared to the much higher values (45-50 KJ mol^{-1}) for E_a of artificial lipid

membranes. Again no phase transition was observed under these conditions.

The fact that activation energies from the osmotic data are low compared with that for water in free solution may be explained by a low degree of water membrane interaction under osmotic stress and compatable with the phenomenon of molecular slippage as suggested by Vieira et al (1970).

The specific sulphydryl crosslinking reagents pHMB and pCMBS showed opposing effects on the activation energies and water exchange times for both human and eel erythrocytes causing an increases in values for E_a in human cells, suggesting water attenuation, and a decrease in those for eels, suggesting a flow amplification.

The effects of mercurial compounds on eel and human cells are explained in terms of conformational changes in the geometry of the aqueous channels leading to their virtual closure (in human) or opening (in eel) through the specific reaction of pHMB or pCMBS with sulphydryl groups in membrane proteins participating in channel structure.

In confirmation of the E_a values for eel and human cells, pCMBS generates similar opposing effects on osmotic water flow. It inhibits the water movement across the human erythrocyte membrane while accelerating it in eel cells. The addition of cysteine reverses both these effects.

The inhibition in human cells brought about by pCMBS treatment is temperature dependent with greater inhibition at 15°C than that at 37°C.

The differing degrees of recovery brought about by cysteine compared to those by bovine serum albumin suggests two classes of membrane sulphydryl groups on both eel and human erythrocytes differing in their location and function in controlling water permeation. The internal groups are responsible for 90% acceleration in eel and 20% inhibition in human cells. The externally located sites are responsible for 80% of water control in the case of human cells.

The use of NEM showed that the sulphydryl groups responsible for water control of both cell types are NEM-insensitive. Treatment by NEM on its own has an insignificant effect on water permeation.

The application of pCMBS, after treatment of eel and human cells with the double bond saturating compound, thiolacetic acid, intensifies the water acceleration effect on eel and attenuates the water inhibition effect on human. This may be attributed to conformational changes destabilizing the membrane. It also suggests that an association of sulphydryl groups with double bonds at strategic sites (eg membrane pores) would be highly effective in controlling membrane permeability. Whether this could form the basis of a natural water controlling mechanism is not possible to predict on the data available but there is no doubt that it is a rapidly acting system.

The nature of ^{203}Hg-pCMBS binding to both intact eel and human erythrocyte membranes are significantly different. In human cells there is desorption of the agent after maximum binding reached 30 minutes post addition. In the case of eel, the maximum binding is reached rapidly (10 min) without subsequent desorption.

The level of desorption by cysteine and bovine serum albumin 30 minutes post ^{203}Hg-pCMBS treatment showed that the distribution of membrane sulphydryl groups is different in both cell types. They are equally divided on both sides of eel red cell membrane, while for human 80% of pCMBS sensitive groups are located internally (close to cytoplasmic surface) and the rest near to the outer surface.

In NEM-treated human cells, the ^{203}Hg pCMBS binds to the NEM-insensitive SH groups which are located externally. In the case of NEM-treated eel red cells it was found that the NEM-insensitive groups are located internally. It can be concluded that the water permeation controlling sites are internal in eel cells and external in human cells.

The pattern of effects brought about by pCMBS on the ion movement across both eel and human erythrocytes membranes is similar. There is Na^+, Cl^- and water gain paralleled by a K^+ loss. The ion movements are faster in eel than human. After three hours incubation for eel cells there is K^+ loss and Na^+ and Cl^- gain to such an extent that there is an increase in cellular ion and hence osmotic content bringing water into the cells. In human cells there is a greater K^+ loss than Na^+ gain and little change in the Cl^-

level. With the slight decrease in cellular ionic content of these cells the water volume does not change for 6 hours post pCMBS treatment. Because of the greater movement of Na relative to K in eel cells, the possibility of Na/K pump reversal is suggested. This is an important concept requiring further investigation.

With the addition of cysteine to both eel and human red cells the loss or gain of ions are slowed, paralleled with a slowing down of water movement across eel cells. The treatment of eel erythrocytes with cysteine up to 1.25 hours post pCMBS addition results in the effective recovery of ion and water balance. The addition of cysteine to human cells 4 hours post pCMBS treatment fails to produce recovery while its addition up to 2.5 hours can restore recovery without lysis.

Pre-incubation of eel erythrocytes with ouabain or furosemide does not prevent the effect of pCMBS on ion and water movement at 15°C.

The exposure of eel and human red cells to increasing doses of ionizing radiation results in a disappearance of membrane -SH groups, paralleled with an increase in water permeation. Both the -SH group disappearance curves and water uptake are consistent and support the suggestion of two classes of membrane sulphydryl groups. It appears that the human erythrocyte membrane -SH groups are more radiosensitive than those of the eel as judged from their respective D_{37} values.

No more water permeation occurs when pCMBS is added to irradiated eel cells suggesting that both agents affect the same -SH groups

responsible for changes in membrane permeability.

The present study shows also that eel erythrocytes have a more effective radiation recovery response than human cells. This fact is apparent from the incomplete -SH reappearance in human compared to complete reappearance in eel under similar conditions. It also appears that the recovery response is an energy demanding mechanism requiring the presence of glucose and elevated temperatures. Like pCMBS, exposure to ionizing radiation produces K^+ loss and Na^+, Cl^- and water gain in human red cells 24 hr post exposure. In the case of eel cells although there is K^+ loss and Cl^- and water gain there is a low Na^+ level in eel cells 24 hours post irradiation suggesting minimal gain of this ion. It may be relevant that for eels, radiation produces a haemolysis resistant red cells that can survive 6 days post exposure at 12°C with less than 15% haemolysis, where for human, all cells are lysed within 48 hours post irradiation.

Damage to mature erythrocytes plays a significant role in the radiation syndrome and it is therefore likely that the recovery processes observed are of great importance for the normal physiology of the erythrocytes. In view of the established role of -SH groups in the maintenance of structure and function of the erythrocyte membrane, it seems possible that the repair process involving membrane -SH groups is critical to erythrocytes under various physiological and pathological conditions. It has been suggested that haemolysis and destruction of erythrocytes in the normal ageing process may result from a loss of membrane sulphydryl groups. Conceivably the ability to reduce membrane SH groups might be important for erythrocyte survival.

Possible future development of the work

Based on the data from chapter 2, a systematic search for the suggested plasma factors influencing membrane permeabilities should be high on the priority list. So much fundamental research on red blood cells has been conducted on washed cells and it became apparent during the course of this work that the simple act of washing and resuspending in physiological buffers significantly influences membrane integrity with respect to transport mechanisms. Preliminary experiments ruled out proteins and divalent ions as controlling influences and perhaps attention should be focussed on catecholamines as known agents that alter the water and ion status of lower vertebrate cells.

With the exception of the extensive data on human cells, there is little information on the number, distribution and chemical characteristics of membrane sulphydryl groups of erythrocytes from other animal species. For a better physiological understanding, from an evolutionary point of view, this gap in our knowledge should be filled. Based on the findings of this work on how the eel's water controlling sites differ from human cells, a general survey, using the techniques developed for mammalian cells would prove particularly productive in cells from animal species having novel adaptational strategies to, for example, water deprivation and low temperatures. Particular examples would be animals in arid environments or those undergoing hibernation in which normal body temperatures fall to 4°C.

Membrane permeabilities are disturbed by -SH binding compounds and this readily suggests investigations on the extent of the role of -SH containing proteins on other transporting mechanisms - such as those for Ca^{++}, amino acids and carbohydrates. Of particular interest in lower vertebrates would be the relationship between catecholamines, water permeability and the involvement of SH proteins.

The fact that pCMBS can rapidly change intracellular ion levels in eel cells and is instantly removed on the addition of cysteine allows an investigation into the role of ion contents and gradients on the mechanisms of cell volume control of hypotonically swollen cells. Using pCMBS, it is possible to control to a degree of precision, the cation content of cells and therefore ask at what level must the (K^+) ion fall before the K/Cl cotransport system fails to operate? The hint of a Na/K pump reversal under pCMBS treatment as an explanation of the rapid water gain seen in eel cells similarly deserves further investigation.

Proton diffusion using NMR relaxation times is a powerful experimental technique that can be used on whole cells measuring diffusion times in milliseconds. It is, however, sophisticated and expensive in its hardware requirements and is highly dependent on instrument availability because of the long scanning times required.

Inevitably with a busy multiuser machine, the work conducted in this project had to be limited, but its advantages in examining cells not under osmotic stress and suspended in their own plasma became obvious and make it a particularly useful technique. It is

unfortunate at present that it relies on the use of relatively high concentrations of the paramagnetic ion Mn^{2+} which has been shown to interfere with normal permeabilities. The search for organic, non-permeant paramagnetic compounds is necessary and apparently feasible for biological research and would give more confidence in the diffusional data than that obtained by present techniques.

Appendix

8. APPENDIX

8.1 E.C.V. Program

The following programs in BBC Basic were used routinely to aid computation of intracellular water and ion contents.

```
,O
>L.
    5 REM CELLWATER
   10 REM CELL WATER CONTENT
   20 PRINT "Weight of empty vial in gm "
   30 INPUT totwt
   40 PRINT "Weight of vial + wet pellet "
   50 INPUT wetwt
   60 PRINT "Weight of vial + dry pellet "
   70 INPUT drywt
   80 LET drycell = drywt - totwt
   90 LET cellwt = wetwt - drywt
  100 PRINT "DPM from 40 ul of pellet extract"
  110 INPUT actpel
  120 PRINT "DPM from original solution "
  130 INPUT actsol
  140 PRINT "Background DPM "
  150 INPUT bgd
  160 LET pelact = actpel - bgd
  170 PRINT " Haematocrit as % "
  180 INPUT htct
  190 LET H = htct * 10
  200 LET solact = actsol - bgd
  210 LET Z = pelact*250
  220 LET Y = Z/solact
  230 LET X = Y*100
  240 LET E = X/H
  250 LET xtrawt = E *cellwt/100
  260 LET corrwt = cellwt - xtrawt
  270 LET V = 1000 * corrwt/drycell
  280 PRINT 'Water content = ";V "ml per Kg d.c.s."
  290 GOTO 20
```

```
>NEW
 10 'REM ECV CORRECTION
 20 PRINT "HAEMATOCRIT AS %"
 30 INPUT htct
 40 LET H=htct*10
 50 PRINT "SAMPLE VOLUME IN ml"
 60 INPUT S
 70 PRINT "CONCENTRATION OF ION IN ECV AS mM/l"
 80 INPUT I
 90 PRINT "MOLECULAR WEIGHT OF ION"
100 INPUT M
110 PRINT "DILUTION FACTOR FOR PHOTOMETER"
120 INPUT F
130 PRINT "DPM from 40ul of pellet extract"
140 INPUT actpel
150 PRINT "DPM from original solution"
160 INPUT actsol
170 PRINT "Background DPM"
180 INPUT bgd
190 LET pelact = actpel - bgd
200 LET solact = actsol - bgd
210 LET Z = pelact*250
220 LET Y = Z/solact
230 LET X = Y*100
240 LET E = X/H
250 PRINT "FLAME PHOTOMETER READING AS mg/l"
260 INPUT D
270 PRINT "WEIGHT OF EMPTY VIAL"
280 INPUT empt
290 PRINT "WEIGHT OF VIAL + DRY PELLET"
300 INPUT dry
310 LET W=dry - empt
320 LET A=htct*S/100
330 LET B=A*E/100
340 LET C=I*B/1000
350 LET G=D*F
360LET J=G/4000
370 LET K=J/M
380 LET L=K-C
390 LET N=L*1000/W
400 PRINT "CONCENTRATION OF ION IN CELLS = ";N " mM/Kg d.c.s."
410 PRINT:PRINT
420 PRINT "ECV = ";E
430 GOTO 130
```

8.2 Electron Microscopy

Electron microscope prepartion was made on blood cells suspended in normal physiological solutions and examined by Jeol 100 CX transmission electron microscope with camera

Sample preparation:

1. Fixation:

1 hr in a 2% solution of OSO_4 buffered with cacodylate to pH 7.4. Cells were washed with 0.9% NaCl solution several times to remove the osmium.

2. Dehydration:

Cells were slowly dehydrated by passage through ethanol in water (30, 50, 70 and 100%). 10 minutes were allowed for each percentage of ethanol followed by three washes in 100% ethanol.

3. Embedding:

Dehydrated cells were mixed with propylene oxide and suspended in a solution of 20% Taab-n resin in propylene oxide for 18 hrs. After one further change in 20% resin, the embedding was continued, using increasing concentrations of 50% and 75% resin over 24 hours.

4. Sectioning:

100 nm light silver to dark grey sections were cut with a Reichert-Junglet $OMCL_3$ microtome, using glass knives. It was found that resin-embedded blood cells were strong enough to mount on grids without a supporting membrane.

5. Staining:

Ultrathin sections of embedded material were stained and exposed for 10 min to uranyl acetate in 70% ethanol. They were then washed in distilled water and further stained with Renold lead citrate for a further 10 min. Stained sections were then observed and photographed with the Jeol 100 CX transmission electron microscope.

6. Notes:

Electron microscopy was a very minor part of this research project but its use gave insight into the technique and allowed the identification of mitochondria (plate 1 p 245) as morphological support of the cell's need for ionic and water balance being dependent on aerobic metabolism.

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